EVALUATION OF PREFRONTAL ISCHEMIC LESIONS ON HIGHER ORDER COGNITIVE BEHAVIOURS IN THE RAT

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ABSTRACT

Stroke is the most common cause of disability worldwide, and it is expected that the global number of stroke survivors will increase significantly over the coming decades. Many survivors of stroke have both short and long-term deficits in function, the most common of which are motor deficits. However, an often forgotten aspect of stroke is its negative and often long-lasting effects on cognitive function. At the moment, there are some effective intervention strategies for treating motor dysfunction post-stroke, but we currently have very limited strategies for treating cognitive dysfunction. Furthermore, there are very few stroke models specifically focusing on higher-order cognitive dysfunctions, also known as executive functions, which have been shown to be affected in a substantial portion of stroke survivors. This makes it difficult to effectively study the effects of potential therapies to alleviate post-stroke cognitive dysfunction. It is also known that the seat of higher-order cognitive function is the prefrontal cortex (PFC), and that damage to this region or by damaging connections to the area can result in executive dysfunction. Therefore, the goal of this thesis is to develop new animal models of post-stroke cognitive dysfunction, targeting specific regions of the rodent PFC. The overarching hypothesis of the project was that injections of the vasoconstrictor, endothelin-1, into particular regions of the prefrontal cortex will cause ischemic lesions in this area, as well as measureable cognitive deficits in higher-order cognitive functions. The overall objectives of the thesis were:

1. To refine and evaluate the effects of endothelin-1 injections into the prefrontal cortex on brain lesion size, and initial behavioural effects in the rat.
2. To measure the effects of refined, focal ischemic lesions within the medial prefrontal cortex on aspects of set-shifting and temporal order memory, both of which are higher-order cognitive functions, in the rat.
3. To further evaluate the model using endothelin-1 to create focal lesions in the orbital prefrontal cortex versus lesions in the medial prefrontal cortex to assess its effects on decision making in the rat.

The initial modelling of post-stroke cognitive dysfunction was attempted using the compound endothelin-1 (ET-1), an endogenous polypeptide capable of constricting blood vessels in the brain. Two separate medial prefrontal cortical (mPFC) ET-1 injection protocols (four injections versus two injections into the mPFC) in the rat were examined in an attempt to understand the size and location of the resulting brain lesion. It was found that the two injection protocol, as compared to the four injection protocol, resulted in a more targeted lesion in the mPFC and resulted in fewer animal mortalities. As well, it appeared that mPFC lesions caused anxiogenic behaviours in these animals as assessed through the elevated plus maze, indicating that these lesions were having some effects on post-stroke cognitive behaviour. Further experiments with the two-injection endothelin-1 model previously established assessed for deficits in specific higher-order cognitive domains, specifically set-shifting and temporal order memory. This was done using a modified set-shifting maze and a temporal object recognition task. It was found that lesions to the medial prefrontal cortex caused specific context-dependent set-shifting dysfunctions in stroke animals. However, attempts to determine the lesion's effects on temporal order memory did not function as expected, indicating

that the methods used to assess this behaviour need to be modified in the future. Finally, two separate models of prefrontal stroke targeting the medial and orbital prefrontal cortices were both examined for their effects on decision making and inhibitory control as assessed through a delay discounting maze, wherein animals would have the choice between an immediately available, but small, food reward or a larger food reward, to which access was delayed. As well, during this testing the animals' ultrasonic vocalizations, which can indicate affective or aversive emotional states, were also recorded while the animals were anticipating a food reward. What was found was that lesions to the orbital, but not the medial, prefrontal cortex caused rats to choose the lower food reward as compared to control animals. Interestingly, only the medial prefrontal lesioned animals changed the relative frequency and duration of their ultrasonic vocalization calls post-stroke, indicating that changes in animal vocalizations are not necessarily correlated to changes in behaviour.

Overall, the work presented in this thesis suggests that specific higher-order cognitive dysfunctions can be accomplished using endothelin-1, thereby providing a potentially new model for the study of post-stroke cognitive dysfunction.

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ABBREVIATIONS

Abbreviation	Term
5CSRTT	5 choice serial reaction time task
ACA	anterior cerebral artery
ACC	anterior cingulate cortex
aCSF	artificial cerebral spinal fluid
ANOVA	analysis of variance
A/P	anterior / posterior
ATP	adenosine triphosphate
CA1	*cornu ammonis* 1
CBT	cognitive behavioural therapy
CIMT	constraint induced motor therapy
D/V	dorsal / ventral
dlPFC	dorsolateral prefrontal cortex
DPI	dots per inch
ED	extradimensional
EPM	elevated plus maze
ET-1	endothelin-1
ETA	endothelin A receptor
HRA	high reward arm
ID	intradimensional
ITI	inter-trial interval
LRA	low reward arm

MCA	middle cerebral artery
MCAo	middle cerebral artery occlusion
MCI	minor cognitive impairment
M/L	medial / lateral
µl	microlitre
mm	millimetre
MMSE	mini mental state exam
MoCA	Montreal cognitive assessment
mPFC	medial prefrontal cortex
MWM	morris water maze
NIHSS	national institutes of health stroke scale
oPFC	orbital prefrontal cortex
PFC	prefrontal cortex
PSD	post-surgery day
RAM	radial arm maze
r-TPA	recombinant tissue plasminogen activator
SSRI	selective serotonin reuptake inhibitor
SST	set-shifting task
SEM	standard error of the mean
SPSHR	stroke prone spontaneously hypertensive rat
TOR	temporal object recognition
USA	United States of America
USVs	ultrasonic vocalizations

vmPFC	ventromedial prefrontal cortex
WCST	Wisconsin card sorting test

CHAPTER 1

GENERAL INTRODUCTION

OVERVIEW

Stroke is a very common acute neurological injury that affects over 50,000 Canadians per year, having devastating effects on both the lives of those afflicted and their families and friends. When people think of the effects of stroke in a general sense, it is thought of in terms of deficits and weaknesses in movement, which are indeed the most common post-stroke deficits (Lawrence et al. 2001). However, some of the overlooked deficiencies following stroke are the cognitive changes that occur when certain areas of the brain are damaged as a consequence of improper blood flow. These deficits can be long-lasting and have detrimental effects on the quality of an individual's life, which can have important clinical outcomes which can include increased recovery time post-stroke and increased incidence of depressive episodes (King 1996; Nys et al. 2006). These deficits in cognitive function, an understandably broad category, can include deficits in learning and memory, language difficulties, emotional incontinence, problems with goal-oriented behaviour, disinhibitory behaviour, and other higher order cognitive deficits. These cognitive deficits, although less common than motor deficits, do occur frequently post-stroke.

One of the unfortunate aspects about acute stroke treatment and why so many are left debilitated is the lack of pharmacological and behavioural interventions that can be administered during or shortly after the stroke. One of the only therapeutic interventions available to clinicians in North America, recombinant tissue plasminogen activators (r-tPAs), has an effective time window of 3-6 hours post-stroke, depending on various circumstances. Clot-busters can only be used during an ischemic rather than hemorrhagic insult and severely restricts its use as tPA can only be used after the patient

arrives to a medical facility and undergoes a CT scan (or MRI) and clinically-verified as ischemic. As such, valuable time from onset to intervention is lost as inadvertent use of tPA on a hemorrhagic stroke will cause more bleeding and worsen clinical outcomes.

In a broad sense motor dysfunctions post-stroke, although debilitating, have the capacity to be treated or minimized through a number of therapeutic options. Some of the most common forms of motor therapies, physical and occupational therapy, have been used with a notable degree of success (Steultjens et al. 2003). As well, a relatively new and notable therapy is known as constraint induced movement therapy, which has been demonstrated to work in both animal models of stroke as well as to help restore limb function in patients (McIntyre et al. 2012; Schmidt et al. 2014). In contrast, there has been very limited success for the treatment of cognitive dysfunction that has occurred due to stroke, and this treatment has been specific to only select subsets of cognitive functions (Cicerone et al. 2005; Chung et al. 2013). Another issue that is faced with regards to stroke and cognitive function relating to treatment therapies, is the lack of ischemic models that demonstrate replicable higher-order cognitive deficits (Jiwa et al. 2010). If we are to treat post-stroke cognitive dysfunctions, we need to be able to model them effectively.

Therefore, the overall aim of the work contained in this thesis is to contribute to the development of a new animal model of stroke damage that will successfully mimic some of the higher-order cognitive deficits that are seen post-stroke. Specifically, this work describes the preliminary modelling of post-stroke ischemic cognitive dysfunction in the rat, which has the potential to be further refined and used for the development of

behavioural or pharmacological therapies for the treatment of post-stroke cognitive dysfunction.

The purpose of this first chapter is to provide background regarding the disease of stroke, how it causes damage, what sorts of deficits result, and the current state of research regarding cognitive dysfunction post-stroke.

1.1 STROKE

The disease of stroke is a non-communicable ailment resulting from the poor flow of blood to the brain, ultimately culminating in damaged brain tissue and cell death. Although the relative rate of death from this disease, and cardiovascular disease in general, has been decreasing in many parts of the Western world since the second half of the 20th century, the effects of this disease can still be widely felt (Sarti et al. 2000). The overall death rate from stroke varies, but roughly one quarter to one third of individuals who suffer a stroke die within a short amount of time post-insult (Anderson et al. 1994; Weimar et al. 2002; Hollander et al. 2003). Despite improvements in death rates, much of the insidiousness of stroke comes from the number of individuals that are left temporarily or permanently disabled post-insult. It has been demonstrated that approximately one third of stroke survivors continue to have deficits in function five years post-stroke, and one seventh of these survivors are permanently institutionalized (Hankey et al. 2002). In another study examining the effects of even minor ischemic events, described as patients with minor functional deficits as assessed by the National Institutes of Health Stroke Scale (NIHSS) score, almost one third of patients continued to have substantial deficits in function (Khatri et al. 2012). In Canada, it has been estimated that approximately 400,000 individuals experience the ongoing effects of stroke, which equates to little over 1% of the total population of the country (Krueger et al. 2015). In other higher-income countries, this trend is similar (Feigin et al. 2014; Mozaffarian et al. 2014).

However, not only is this disease having a profound effect in developed countries, but currently developing countries with less robust health-care systems are also burdened by stroke, and the rates of stroke are increasing in these areas (Feigin et al. 2009). The economic and social burden of this disease is also deeply felt. In Canada alone, stroke has been estimated to cost 2.9 billion dollars annually in direct medical costs and other indirect costs (Mittmann et al. 2012).

The risk factors associated with ischemia are numerous, and include hypertension, current smoking status, waist-to-hip ratio, diet, exercise, alcohol intake, stress, and genetic factors (O'Donnell et al. 2010; Traylor et al. 2012). Although the relative number of people smoking in Canada and many other countries is decreasing (Ng et al. 2014), Canadians on the whole are gaining weight (Luo et al. 2007), exercising less than they should be (Colley et al. 2011), and getting older (Statistics Canada 2014), which has the potential to affect the rates of stroke in Canada in the coming decades.

Strokes can be subdivided into two major categories, ischemic strokes and hemorrhagic strokes. Ischemic strokes involve a reduction of blood flow in vessels in the brain, and hemorrhagic strokes are the result of a ruptured blood vessel in the brain. The work contained within the following chapters of this thesis focuses on the effects of ischemic stroke rather than hemorrhagic stroke, therefore a brief discussion as to what ischemia is, and its cellular and molecular effects on the brain, will be given.

1.2 ISCHEMIA

As mentioned previously, the most common form of stroke is the result of an ischemic event, which can be caused by an embolus lodged within the vessel, a thrombus having developed within the artery, or a lack of blood flow to the brain caused by some other event external to the brain. These events result in a reduction of blood flow, and this reduction can become acutely damaging at flow rates below 35 mls per 100 g of tissue per minute (Bandera et al. 2006) . Because of this, a lack of glucose and oxygen from the blood to brain cells ultimately leads to a cascade of damage within the brain.

When comparing the relative rates of ischemia to other forms of stroke, notably hemorrhagic stroke, ischemia occurs much more commonly. It has been estimated that ischemic strokes form the majority of all strokes in the United States, occurring in approximately 87% of all stroke cases (Mozaffarian et al. 2014), and values reported previously in Canada appear to be similar (Mayo et al. 1996). As ischemia is the most prevalent form of stroke, and the animal models discussed in subsequent chapters of this thesis model damage caused through ischemic mechanisms, how ischemia causes damage will now be discussed further, with a basic summary presented in Figure 1.1.

1.2.1 Mechanisms of Ischemic Damage

To understand how ischemia causes damage in the brain, the first issue that must be discussed is the different regions of damage that occur due to ischemia. Ischemic

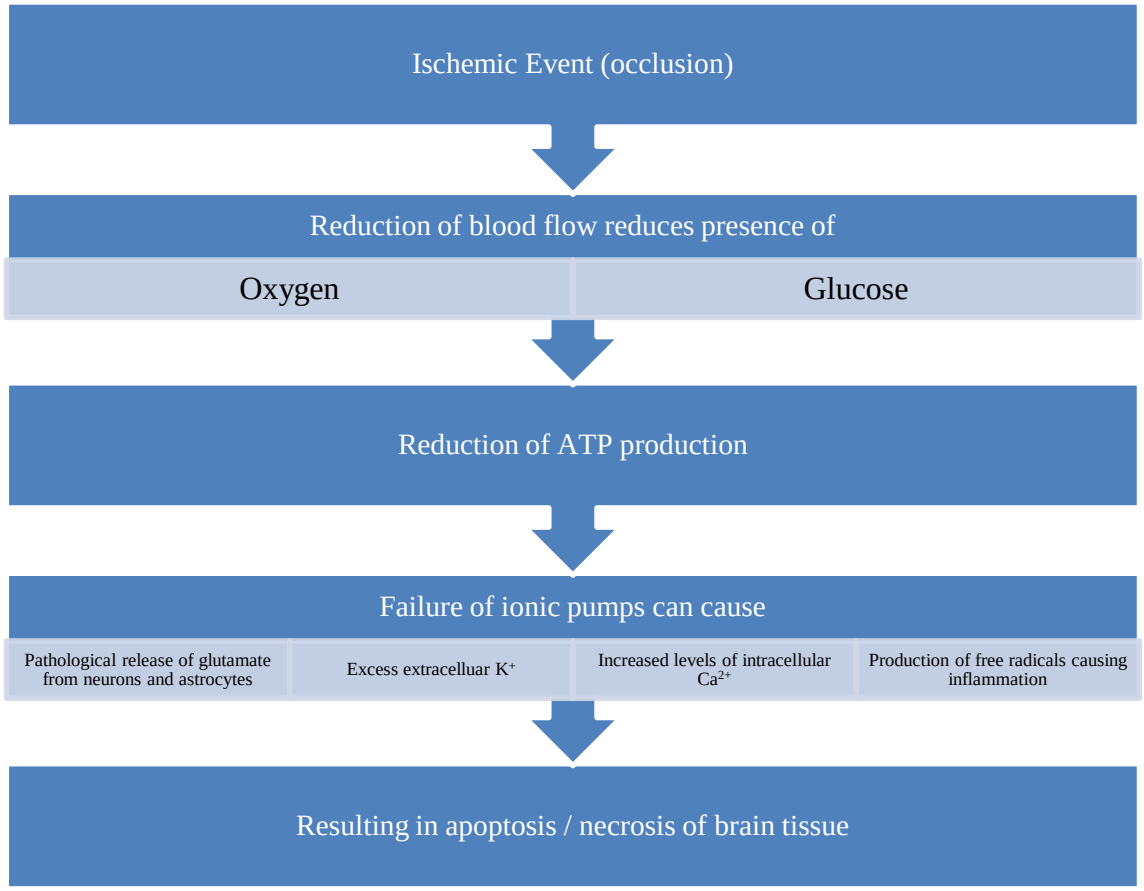


Figure 1.1 Basic overview of mechanisms of ischemic damage
Adapted and simplified from Durukan and Tatlisumak (2007)

damage can be subdivided into two major areas: the core of the infarct and the penumbra (Astrup et al. 1981). These two regions can be differentiated by how much blood these areas receive during an ischemic event, how quickly these two regions succumb to reduced blood flow, and by what mechanisms the cells die (Kaufmann et al. 1999; Broughton et al. 2009; Yuan 2009).

In brief, the core of the infarct is the area that receives little to no blood flow as a result of the ischemic event. Unlike many tissues in the body, which can survive ischemic conditions for a comparably longer period of time (Jaeschke and Lemasters 2003; Basile et al. 2011), ischemic conditions in the brain quickly result in damage. As a result of this lack of blood flow, the cells in the brain can die relatively quickly, and can perish within minutes during acute injury (Lee et al. 2000).

The core of the infarct is different from the ischemic penumbra, which is an area that receives more blood flow than the core, but may still be susceptible to short or long-term damage depending on a few different circumstances which can include the amount of blood flow, the type of neuronal cells involved, the occurrence of spreading depression following insult, and its susceptibility to reperfusion injury (Jenkins et al. 1981; Dijkhuizen et al. 1998; Lee et al. 2000; Hinzman et al. 2015).

1.2.2 The Ischemic Cascade

The ischemic cascade is the series of interlinked events that ultimately culminate with the death of neuronal tissue in the central nervous system. Ultimately, the primary issue at play with the ischemic cascade is the lack of adenosine triphosphate (ATP)

within the brain tissue, which is the result of insufficient production of this molecule caused by a lack of nutrients reaching these cells (Xing et al. 2012). The lack of ATP production leads to a loss of ionic homeostasis, as the cellular mechanisms responsible for the addition and/or removal of Na^+, K^+, Cl^-, and Ca^{2+} no longer function. This leads to pathological influxes and effluxes of these ions into and out of the cytoplasm (Rothman and Olney 1986). These events further precipitate downstream effects, which can have detrimental effects both for these cells undergoing this ionic imbalance as well as other nearby cells.

The beginning of the ischemic cascade can be understood by knowing that cells within the brain function almost exclusively through the production of ATP, produced by the breakdown of glucose to gain the energy found within its chemical bonds. Most other mechanisms of energy production (including lactic acid production), as well as energy storage in the form of glycogen, are not nearly as prevalent in the brain compared to other areas of the body (Brown and Ransom 2007). Because of this, the brain needs a consistent supply of glucose, as well as oxygen, to survive.

Once the brain has used up its minimal stores of glucose and oxygen during an ischemic event, the ionic balances that neurons need in order to survive begin to break down. To give a sense of the importance of proper ionic balance in the brain, one of the primary molecules that contributes to this in neurons, the sodium-potassium pump, has been estimated to use over 40% of the available oxygen in the rat brain under normal conditions (Clausen et al. 1991). As these molecules are no longer available to perform their functions under ischemic conditions, potassium begins to leak from the cell and sodium, along with chloride, begin to enter (Jiang et al. 1992).

This loss of ionic balance, which leads to the depolarization of neurons, can cause the release of the excitatory neurotransmitter, glutamate. Glutamate's release from the presynaptic neuron is dependent on calcium influx into the cytoplasm. From there, glutamate binds to ionotropic glutamate receptors on the post-synaptic neurons and depolarizes these cells (Sharma and Vijayaraghavan 2003). Too much depolarization within the infarct area over a short period of time can lead to pathologic excitatory conditions in the brain, which can ultimately cause cell death (Hinzman et al. 2015).

Inflammatory processes are also activated during and after the ischemic event, and can also damage parts of the brain (Zheng and Yenari 2004). Much of this inflammatory damage can be done through calcium, which activates the inflammatory system. Calcium again acts as a second messenger and upregulates a number of cytokines present through the action of NfκB, a known transcription factor (Clemens et al. 1997; Zheng and Yenari 2004). This further production of cytokines can directly lead to cell death in some cases, but can also be involved in the recruitment of phagocytic cells which also play a role in neuronal death (Neher et al. 2013).

Other events in stroke that lead to cell death in the peri-infarct zone include apoptosis, a form of programmed cell death, which is mainly triggered by the influx of calcium caused both by the glutamate release from neurons as well as the damage induced by the anoxic conditions surrounding the cell (Nicholson et al. 1977). All of this accumulated cellular damage caused by ischemic conditions in the brain, whether it was within the core or the penumbra of the insult, may ultimately lead to deficits in function, as detailed in the following section.

1.3 DEFICITS IN FUNCTION POST-STROKE

Because of the aforementioned cellular damage that is incurred due to ischemia, people can suffer functional deficits in a multitude of modalities that affect everyday life. The length of time that these deficits last can be short term, lasting a timeframe of days to weeks. This is because many smaller stroke injuries can be repaired through a variety of different cellular and molecular mechanisms that occur post-stroke, including the formation of new synapses (Jones and Schallert 1992), an increase in the number of dendritic spines (Kolb and Gibb 1993), increased expression of growth factors (Talwar and Srivastava 2014), and the growth of new neurons in particular parts of the brain (Liu et al. 1998). The difficulty is that very often there is not a full spontaneous recovery of function, even after the brain's repair and growth mechanisms have been activated. The rates of full spontaneous recovery of function in the brain appear to depend on both the initial amounts of damage that have occurred in the brain as well as the location of these lesions (Desmond et al. 1996; Vogt et al. 2012).

If these deficits disappear without intervention, they are said to have spontaneously recovered. Depending on the nature of the deficit, some spontaneous recovery without intervention can generally be seen with motor deficits for the first one to three months post-stroke, after which time recovery without intervention is limited (Bonita and Beaglehole 1988; Duncan et al. 1994). Some similar timeframes are seen for cognitive deficits, although the window of spontaneous recovery has been shown to be slightly longer than that in motor deficits (Wade et al. 1988; Chemerinski et al. 2001). Unfortunately, spontaneous recovery of deficits in function does not occur

equally for everyone, with those with mild deficits often regaining full capacity of function, and those with more severe deficits being unable to live independent lives (Cramer 2008). Factors that can affect rates of spontaneous recovery in stroke can include age (Zhang et al. 2005), size and location of infarct (Rogers et al. 1997; Plowman et al. 2012), and the experimental method by which the damaged was caused (Voorhies and Jones 2002), among others. As well, not only does spontaneous recovery not always happen, but some individuals with stroke may actually see some declines in function over time (Tatemichi et al. 1993).

Because the disease of stroke affects many different aspects of behaviour and overall function, its effects will be broken down into three separate categories: motor dysfunction, sensory dysfunction, and cognitive dysfunction.

1.3.1 Motor Dysfunction

Motor dysfunction incorporates many separate disabilities in movement, and can include weakness in one side of the body (hemiparesis), complete loss of use of movement (paralysis), and spastic or uncoordinated movements of voluntary muscles (Bonita and Beaglehole 1988; Ward 2012) . Hemiparesis is the most common form of motor disability post-stroke, and can occur in approximately 80% of stroke patients (Granger et al. 1988). Overall, the number of people who survive the initial insult and have ongoing motor deficits is approximately 25% (Bonita and Beaglehole 1988).

Despite the number of challenges faced by people with ongoing motor disabilities post-insult, there have been significant improvements in the development of

successful recovery treatments to alleviate motor dysfunction. One of the best known of these treatments, constraint induced movement therapy (CIMT) has proven to be very effective in hemiparetic patients (Wolf et al. 2006), although . Other forms of motor therapy that are commonly employed include task-specific training as well as providing environmental enrichment for patients in recovery (Takeuchi and Izumi 2013). These forms of treatment for motor dysfunction post-stroke, although the disease is still debilitating for many patients, can offer the promise of full functional recovery.

1.3.2 Sensory Dysfunction

The loss of sensory function that occurs post-stroke is thought to be quite common, estimated to occur in approximately half of the individuals and affects many different types and aspects of sensory function. However, one study indicated that over 80% of patients, one week post-stroke, had sensory dysfunction in at least one sensory modality (Kim and Choi-Kwon 1996). Overall, tactile sensation appears to be the modality of sensory function that is most affected by stroke, although proprioception can also be affected (Tyson et al. 2008). Although there is still much work to be done in the domain of sensory dysfunction and restoring function, much progress has been made regarding experimental treatments for this aspect of the disease. The evidence regarding its treatment has been well summarized by a review by Sullivan and Hedman (2008), and includes a large variety of treatments including sensory discrimination tasks, electrical stimulation, and intermittent pneumatic pressure.

1.3.3 Cognitive Dysfunction

Although not as prominent or as common as motor dysfunction, cognitive dysfunction post-stroke is still a major concern. Post-stroke cognitive dysfunction has been estimated to occur in approximately one third of stroke survivors (Zhou et al. 2005), however, estimated measurements of cognitive dysfunction are dependent on the types of tests used, as well as determining the degree of impairment needed to qualify as a deficit in cognitive function (Sun et al. 2014). Therefore, depending on the type of test used, estimates as to the number of patients exhibiting cognitive dysfunctions post-stroke can be even higher. For example, it has been shown that in some cases, post-stroke depression can occur in over half of patients recovering from stroke (Kauhanen et al. 1999).

One of the more difficult aspects of cognitive dysfunction is the lack of recovery of function in many individuals affected. In one two-year study by Hochstenbach et al. (2003), it was demonstrated that in a few patients some long-term cognitive improvements were made, but for most patients cognitive dysfunction either did not improve and/or actually worsened over the course of the study. This finding is repeated in a number of other studies demonstrating poor long-term cognitive improvement post-stroke (Desmond et al. 1996; Rasquin et al. 2004). So, although cognitive recovery is possible in a small subset of patients long-term, many do not fully recover cognitive function and others can suffer further cognitive decline.

Emotional dysfunction and disturbances are also common post-stroke of which the most common symptom is depression, reported to occur as either mild or major

depression in roughly one fifth of patients (Brodaty et al. 2007). Emotional dysfunction can also include mania, anxiety, and disinhibitory control, which all occur somewhat less frequently post-stroke.

For the most part, cognitive and emotional deficits are typically assessed post-stroke by a number of neurological tests, including the Montreal Cognitive Assessment (MoCA), the Mini Mental State Exam (MMSE) (Cumming et al. 2013), which are some of the more common cognitive tests used.

Now that the rates of cognitive dysfunction and general categories of these deficits have been explained, specific types of deficits with a concentration on higher-order cognitive deficits will now be discussed.

1.4 TYPES OF COGNITIVE DYSFUNCTION POST-STROKE

As discussed in the previous section, cognitive dysfunction can be a common occurrence in stroke patients. As well, one of the other major issues regarding cognitive dysfunction post-stroke is that it can also affect motor function as well as delay recovery time post-stroke (Tatemichi et al. 1994).

There are a multitude of different types of common cognitive dysfunctions that occur post-stroke, and some of these broad categories will be reviewed below.

1.4.1 Memory Deficits

Depending on the nature of the stroke, individuals often have complications with learning new information as well as retaining and recalling information. Overall, approximately 30% of patients surviving a stroke have some form of memory deficit (Cullen et al. 2007), and the recovery from these memory deficits is not assured. These memory deficits include deficits in both prospective and retrospective memory (Kim et al. 2009), wherein "prospective deficits" refers to failing to be able to remember and plan out and execute new actions, and "retrospective deficits" refer to the recollection of previously acquired memories. Overall, deficits in memory function post-stroke do not appear to be correlated with having more severe strokes, but they are associated with decreased functional capabilities with everyday tasks and can interfere with overall stroke recovery (Wade et al. 1986).

1.4.2 Mood Disorders and Emotional Incontinence

Although perhaps not as common as other types of cognitive deficits, emotional incontinence and mood disorders do occur post-stroke (Robinson 1997). The sorts of deficits included in this category include increased levels of anxiety, which can occur in over one fourth of patients (Barker-Collo 2007), as well as post-stroke mania and chronic fatigue.

Interestingly, although mood disorders may be common post-stroke, many psychiatric issues appear to dissipate within the first year following insult, suggesting

that these symptoms will alleviate themselves over time (House et al. 1991). Despite this, these deficits in function has been reported in a number of patients post-stroke, and affect not only the stroke survivor but also their family, friends, and caregivers, causing them undue burden (Williams 1994).

1.4.3 Post-Stroke Depression

One crucial aspect of cognitive dysfunction that has garnered a lot of attention is depression following stroke. Post-stroke major depression has been reported to occur in as many as one third of all stroke survivors (Gonzalez-Torrecillas et al. 1995), and can occur for a variety of reasons. The first of these reasons is the location of the lesion, with lesions located near the anterior portion of the left hemisphere resulting in more cases of depression (Robinson et al. 1984), although further meta-analysis of the literature of post-stroke depression gives a conflicting view of this hypothesis (Bhogal et al. 2004). Lesion size has also been linked to the severity of post-stroke depression, with larger lesions being associated with increased depressive symptoms (Nys, van Zandvoort, van der Worp, et al. 2005).

The time course of the disease has been reported to run for approximately 1-2 years post-insult (Parikh et al. 1987), and somewhat paradoxically the earlier the depressive symptoms appear following the stroke, the shorter these depressive symptoms can last (Aström et al. 1993).

1.4.4 Post-Stroke Dementia

Dementia, although it is an amalgamation of many forms of cognitive dysfunction, specifically refers to an acquired persistent deficit in overall mental function, with deficits in at least three different spheres of mental function which can include memory, abstract thought, or personality (Cummings 1984). Although Alzheimer's disease is reportedly the most common form of dementia, dementia incurred through one or multiple ischemic events is also prevalent, and it has been estimated that approximately one fifth of people under 65 with dementia acquired it through some sort of vascular event (Harvey 2003). In another study of those 85 years of age, just under half of those with dementia acquired it through some sort of disruption of the vasculature of the brain (Skoog et al. 1993). The risk factors associated with developing this form of impairment post-stroke are similar to the factors associated with having the initial ischemic or hemorrhagic insult (Barba et al. 2000)

1.4.5 Higher-order Cognitive Dysfunctions

Higher-order cognitive functions, also known as executive functions, are a series of top-down cognitive functions related to the overall behavioural flexibility of an organism (Diamond 2013). The types of behaviours associated with these functions include attention and attentional processing, decision making, and goal-oriented behaviour.

Those who have had a stroke often have difficulties paying proper attention to stimuli or features of their environment (McDowd et al. 2003). Other attentional difficulties encountered post-stroke include both the slowing of attentional processing (Hochstenbach et al. 1998), as well as properly being able to switch attentional focus (Vataja et al. 2003). These sorts of deficits can be caused through, or correlated to, damage to certain specific areas of the brain which include the prefrontal cortex as well as frontal-subcortical connections to this region.

Another form of cognitive deficit related to higher-order cognitive dysfunction includes pathological gambling (Cognat et al. 2010), which can be related to improper decision making as well as poor impulse control. From individual case studies, it appears that this effect can be due to bilateral lesions to the basal ganglia, but other stroke lesions are known to cause deficits in proper decision making and poor impulse control (Bechara 2000). Not only are there differences in the types of higher-order cognitive deficits post-stroke, but there are even differences in the types of deficits between the sexes as well, with women becoming more impulsive and displaying more planning deficits as compared to men, controlling for other demographic factors (Scheffer et al. 2011), further complicating rehabilitation post-insult.

Finally, what is especially difficult with deficits in higher-order cognitive functions post-stroke is that they can interfere with the recovery of basic life tasks. For example, deficits in maintaining attention have been shown to be correlated with decreased functional recovery post-stroke, which is probably attributable to the fact that learning motor skills can be dependent on functional attentional processing (McDowd et al. 2003). As well, deficits in cognitive function that result from deep lacunar infarcts

have also been associated with a slower functional recovery, which provides justification for the idea that aiding the recovery of cognitive deficits post-stroke could improve other forms of functional recovery (Mok 2004).

1.5 TREATMENT OPTIONS FOR COGNITIVE DEFICITS

Unfortunately, the current treatment climate for cognitive deficits post-stroke is lacking. This is due to the considerable shortage of effective treatments and therapies to prevent cognitive dysfunction from occurring, or for treating those with long-term cognitive dysfunction post-stroke, although there are a host of new and novel therapies currently in development.

In the past there have been a number of pharmacological agents tested with the express purpose of rescuing brain tissue before it undergoes permanent damage. Unfortunately, virtually all of these pharmacological products failed during some phase of the clinical trials. It has been suspected that many of these drugs have failed because of certain parameters behind the models tested, one of which is that some of the current animal models do not accurately reflect what is seen in human patients (Cheng et al. 2004; Minnerup et al. 2012).

As well, there has been a considerable lack of specifically focused and effective cognitive therapeutics. Although there have been some pharmacological products developed for the treatment of other neurological diseases characterized by cognitive deficits, these drugs are either not effective in the treatment of cognitive deficits post-stroke or have not been extensively tested in order to be approved for treatment. In the

following sections a brief review of the current and experimental treatments used to treat or alleviate some of the cognitive deficits found post-stroke is presented and summarized in Table 1.1.

1.5.1. Current Pharmacological and Behavioural Treatments

The main pharmacological therapies that are used to alleviate and prevent the worst effects of an acute ischemic stroke are recombinant tissue plasminogen activators, or r-tPAs. This family of compounds is quite effective in preventing the worst of damage that occurs from stroke by dissolving the thrombus or embolus that has been lodged inside of the blood vessel (Zangerle et al. 2007). Unfortunately, the use of r-tPAs is not a panacea for stroke treatment for a number of reasons. First, the drug cannot be used for the treatment of hemorrhagic stroke, as the dissolution of blood clots would cause further damage. Secondly, r-tPAs can increase the risk of intracerebral haemorrhage, even in cases of ischemic stroke (Hacke et al. 1998). Third, another aspect of r-tPAs is their relatively small time window for use, which means that only a small minority of patients that could receive this drug end up receiving it at the hospital (Katzan et al. 2004). However, despite these drawbacks, when r-tPAs are used they can be very effective at preventing damage post-stroke.

Another drug that is used in clinics worldwide is MCI-106 (also known as Edaravone under the trade name Radicut ®), which is a free radical scavenger licensed for use in Japan (Lapchak 2010). The drug works through the quenching of HO· radicals formed during the inflammatory response during ischemia, which limits the amount of

Table 1.1 Summary of treatment options for stroke and post-stroke cognitive deficits from section 1.5.

Current Therapies	r-TPAs	↓ infarct size[1]
	Edaravone	↓ amount of free radicals ↓ infarct size
	Behavioural	↓ aphasia, attentional deficits
Experimental Therapies	Cholinesterase inhibitors	↑ cognitive functions, including executive function[2]
	Antidepressants	↓ depressive symptoms[3] ↓ emotional disturbances ↑ spatial memory
	Behavioural	Improvements in some specific cognitive dysfunctions (working memory, post-stroke depression)

[1] Zangerle et al. 2007
[2] Auchus et al. 2007
[3] Li et al. 2009

damage done through inflammation. One of the advantages of Edaravone is that it has a therapeutic window of 24 hours post stroke onset, giving ample opportunity to mediate damage. Although the drug is so far unused in North America to treat ischemic stroke, current safety and efficacy trials are ongoing in an attempt to bring it to market (Kaste et al. 2013).

Many of the behavioural therapies that are implemented for treating cognitive deficits specifically target certain daily activities or functions that a person would employ in everyday life. Unfortunately, the targeted treatments for specific losses in function often cannot transfer to a patient's other daily life activities (Sturm and Willmes 1991; Doornhein and De Haan 1998), which potentially makes the relearned skill applicable only to that cognitive domain and difficult to generalize to others.

One of the major targets of current behavioural treatment is aphasia; a language disorder with symptoms ranging from loss of communicative ability to understanding different forms of communication themselves, and there is much evidence to demonstrate that targeted therapies for the treatment of aphasia have positive effects for patients, the most prominent of which is speech language therapy (Bhogal et al. 2003; Zumbansen and Thiel 2014). Other current cognitive behavioural therapies for stroke include treatment for attentional deficits (Loetscher and Lincoln 2013). Again, one of the issues with cognitive treatments is the transferability of benefits from one area of life to another. This is to say, improving in one cognitive domain does not necessarily imply that one can improve at another simultaneously, which can limit the use of broad-based behavioural treatments.

1.5.2 Experimental Pharmacological and Behavioural Treatments

Because of the lack of current therapies for recovery of cognitive function post-stroke, new methods are being developed to alleviate these incurred deficits in function. Despite many failed clinical trials of pharmacological therapies aimed to treat stroke through a variety of mechanisms (Kidwell et al. 2001), there are new therapies that have shown considerable promise at alleviating the post-stroke cognitive impairments that are commonly found in this disease. At this point, many of these are still in various stages of clinical trials, although a number of drugs previously developed and marketed for the treatment of other neuropsychiatric disorders, have shown some promise in the treatment of post-stroke cognitive dysfunction.

The first family of drugs with some demonstrated effectiveness for post-stroke cognitive dysfunction are cholinesterase inhibitors, which are currently used clinically as treatments for Alzheimer's disease. Two of these, donepezil and galantamine, have demonstrated particular promise. Donepezil has been shown to improve cognitive function in cases of vascular dementia as well as to improve cognitive function in cases of acute ischemic stroke (Black et al. 2003; Barrett et al. 2011). As well, galantamine has demonstrated potential for the improvement of executive functions post-stroke (Auchus et al. 2007) as well as being effective for the treatment of post-stroke aphasia (Hong et al. 2012).

Some current anti-depressant drugs have also shown promise. Experiments using Fluoxetine, a selective serotonin reuptake inhibitor (SSRI) have demonstrated efficacy in the treatment of some cognitive dysfunction, alleviating some of the spatial memory

deficits (Li et al. 2009) as well as emotional disturbances that occur post-stroke (Choi-Kwon et al. 2006). As well, it also appears to function well for preventing the depressive symptoms that occur following stroke, which is to be expected for an SSRI (Yi et al. 2010).

Experimental forms of behavioural therapy that attempt to target overall cognitive function are in the midst of being developed. Unfortunately, at this stage the results have been mixed (Chung et al. 2013; Prokopenko et al. 2013), and it appears that individually targeted therapies for particular cognitive deficits may function best. For example, individual tasks with the aims of strengthening working memory appear to function quite well (Westerberg et al. 2007), as well as new experimental tasks specifically targeting aphasia (Palmer et al. 2012), as mentioned in the previous section.

Other forms of behavioural therapy, including cognitive behavioural therapy (CBT) for depression post-stroke have demonstrated some promise (Broomfield et al. 2011) , but recent studies have indicated that they may not be as effective as previously thought (van Eeden et al. 2015).

As can be seen, many of our current therapies for cognitive dysfunction post-stroke are lacking, and although there are some experimental therapies "in the works", further research is required. One manner by which research into post-stroke cognitive dysfunction could be facilitated would be to increase the diversity of our current repertoire of animal models of post-stroke cognitive dysfunction, thereby giving us innovative means to test novel therapies.

1.6 CURRENT RODENT MODELS OF STROKE DISPLAYING COGNITIVE DYSFUNCTION

There is a long history of modelling brain ischemia, dating back to the early 20th century with global ischemic animals models induced through a variety of mechanisms (Hossmann 1998). This is in contrast to many of the newer models of stroke, which attempt to model the disease through more focal means, done most often by targeting the middle cerebral artery (MCA). However, part of the issue with the current animal models of ischemic stroke is that there is no specific emphasis on modelling the cognitive changes that occur post-stroke, as many of them concentrate on replicating the pathological motor symptoms seen in human patients (Kumar et al. 2016). The issue here, as highlighted in previous sections, is that patients often present with cognitive deficits post-stroke. If research into new treatments is to continue in this domain, new stroke models with a focus on cognitive deficits need to be developed.

In the following section a brief review of the current animal models displaying cognitive deficits, as found in the literature, is presented. Although there are models which focus on neo-natal ischemic stroke, some of which have shown cognitive deficits (Almli et al. 2000; Gonzalez et al. 2009), the focus in the following sections will be cognitive deficits induced in adult animals, with a specific focus on rodent models.

1.6.1 Middle Cerebral Artery Occlusion (MCAo)

The most common form of stroke in human patients is an occlusion affecting brain territory supplied by the middle cerebral artery, which has been estimated to account for approximately two thirds of all ischemic strokes in human patients (Bogousslavsky et al. 1988). The MCA is the largest of the branches of the internal carotid artery, and feeds a significant portion of the brain (Grotta et al. 2015). The vessel is ultimately responsible for sections of all four lobes of the human brain as well as significant anatomical structures within, including the caudate nucleus, the globus pallidus, and large swaths of the cerebral cortex (Tatu et al. 2012).

The technique of specifically occluding the MCA was first developed in the rat by Robinson et al (1975), which was accomplished by permanently ligating the right middle cerebral artery, producing changes in both behaviour and neurotransmitter abundances in select regions. Further refinement of this model was done through the work accomplished by Bederson et al. (1986) as well as Longa et al. (1989) in order to more reliably produce select focal lesions and to accurately describe the regions damaged by this method.

Depending on the exact methods by which the MCAo was induced, as well as the amount of time the MCA is occluded, this surgical procedure in the rat can cause damage to many different structures in the brain. These structures can include portions of the amygdala, hypothalamus, and hippocampus, as well as causing extensive damage to much of the neocortex and striatum (Rubino and Young 1988; Butler et al. 2002; Popp et al. 2009).

The MCAo model offers many advantages to the study of cerebral ischemia in the rat; it can closely mimic human ischemic stroke through the production of motor and sensory deficits (Yonemori et al. 1998; Zhang et al. 2000), it produces a fairly reproducible lesion, and has a relatively low mortality rate (Macrae 2011). As well, the model has demonstrated a significant correlation between infarct volume and motor deficits (Rogers et al. 1997), replicating another facet of strokes found in human patients (Alexander et al. 2010). Though not as commonly observed, animals that have undergone an MCAo can experience some cognitive deficits, including deficits in spatial learning and memory (Wang et al. 2006; Hu et al. 2013).

Although the MCAo model is one of the most prominent models in the literature for studying the pathophysiology of stroke, it is not without its drawbacks. Some of these drawbacks can be due to the methods by which we induce the stroke, and others are simply because of the nature of the model. For example, the use of the intraluminal filament in the temporary MCAo model can run the risk of producing an intracerebral haemorrhage (Schmid-Elsaesser et al. 1998). The intraluminal thread model also runs the risk of producing damage outside of the area of interest, as the thread may block other vessels feeding from the internal carotid artery (Macrae 2011). Microsphere injections into the blood stream intended to occlude the MCA can also cause problems, in that the blockage intended for the MCA can become multifocal and can be found throughout the brain, as well as producing a heterogeneous lesion within the area fed by the MCA (Mayzel-Oreg et al. 2004). Methods for inducing a temporary MCAo that require a craniotomy and the use of vascular clips can produce lesions, but are technically complex and may produce a lesion of variable size (Macrae 2011)

Other drawbacks with the MCAo model include the differences in relative recovery times between rats and human patients. Although the temporary occlusion of the MCA in rodents causes demonstrable deficits in function, many of these deficits are temporary in nature, with some motor functions returning to almost full capacity within one to three weeks (Sun et al. 2008; Liu et al. 2009). In humans, even with therapy, a full recovery from a stroke caused by an MCAo takes considerably longer, if it happens at all (Kwakkel et al. 1999; Miyai et al. 1999)

However, specifically concerning this thesis, one of the biggest disadvantages of this model is the lack of documented higher-order cognitive deficits associated with a rat MCAo (Jiwa et al. 2010), despite the fact that many human patients who have had a stroke can experience higher-order cognitive changes post-stroke (Leśniak et al. 2008; Paradiso et al. 2011). So, although the MCAo model represents an effective method of modelling cerebral ischemia, the drawbacks of the model are well documented and further stroke modelling, especially of cognitive deficits post-stroke, is required.

1.6.2 Bilateral carotid artery occlusion (BCAo)

Bilateral carotid artery occlusion, a method of mimicking stroke cardiac arrest originally developed by Eklöf and Siesjö (1972) involves the occlusion of two of the main arteries that feed the forebrain.

Specifically for studying cognitive deficits post-stroke, one of the main advantages of this model is that certain modifications of the model can induce a variety of cognitive deficits. For example, learning and memory performance in the radial arm

maze has been shown to be affected in this model, most likely due to the damage induced to certain select regions of the hippocampus (Ni et al. 1994; Peng et al. 2007). Furthermore, this model has demonstrated some deficits in higher-order cognitive functions, including deficits in executive functions as measured through reversal learning and attentional set-shifting tasks (Soria et al. 2013).

One of the disadvantages of this model is that it does not represent a focal ischemic event within the brain, but rather it best represents a prolonged state of chronic hypoperfusion culminating in vascular dementia (Farkas et al. 2007). As well, much of the damage precipitated by the ischemic event occurs within the CA1 region of the hippocampus and other deeper brain regions, and can leave much of the rest of the cortex unaffected (Iwasaki et al. 1989). Because of this, the model may be unsuitable to effectively study focal ischemic injury, which is generally the more common form of brain injury due to stroke.

1.6.3 Hypertensive Stroke-Prone Animals

The Stroke Prone Spontaneously Hypertensive Rat (SPSHR) was originally developed by Okamoto and Aoki (1963). This strain of rat has demonstrated some deficits in cognitive function as measured through the radial arm maze (Peng et al. 2005). As well, the model has also demonstrated deficits in spatial and working memory within the Morris Water Maze (Grünblatt et al. 2015).

Although this method of modelling hypertension, and through further experimentation has demonstrated some deficits in executive function in some non-

human primates, (Moore et al. 2002), so far none have demonstrated clear evidence of executive dysfunction for rats or other rodent models. Therefore, it is unclear at this time whether this model simply does not display these behavioural deficits or whether deficits in these behaviours have simply not been adequately explored.

Another disadvantage of the model is that the animals may display these cognitive deficits, not as a result of an ischemic insult but as the result of continuous hypertension, which in other modalities has demonstrated decreased cognitive capacity or dementia without the involvement of a coincident stroke (Kilander et al. 1998; Inaba et al. 2009).

1.6.4 Photothrombosis

Photothrombosis, proposed initially by Rosenblum and El-Sabban (1977) in mice and further refined by Watson et al. (1985), is a method by which infarcts can be induced by photochemically causing platelet aggregation in surface vessels of the brain. The process works through the injection of a photo-active dye (typically a compound called Rose-Bengal) into the blood stream of a live animal. Once the dye has been injected, a cold light beam is shined on the area of the brain that the experimenter wants damaged. This light causes the photo-active particles to produce reactive oxygen species which damage the endothelial cells of blood vessels, leading to the formation of thrombi within the vessels themselves (Labat-gest and Tomasi 2013).

This method has been used very successfully to produce motor deficits in animals, depending on the area that has been targeted (Wood et al. 1996; Lee et al.

2007). In terms of modelling cognitive deficits, this form of stroke has been able to produce some deficits in rodent, and can even mimic progressive cognitive decline post-stroke. For example, a mouse model of progressive cognitive decline induced through photothrombosis directed bilaterally at the sensorimotor cortex induced object recognition deficits as well as persistent deficits in spatial learning and memory assessed through the Morris Water Maze (Schmidt et al. 2015). Along with these findings, rats undergoing this procedure in the sensorimotor cortex also experience difficulties with memory recall and spatial memory (Diederich et al. 2014). In addition, photothrombosis has been used within the prefrontal cortex, leading to changes in behaviour in a conditioned passive avoidance reaction task (Romanova et al. 2002). Interestingly, this model was not tested for changes in other prefrontal cortex-associated behaviours.

This method, like any other, is not without its drawbacks. Because the dye is found equally within all arterial vessels, the light that is shined upon the vessels will affect each vessel relatively equally. Because of this, the properties of the infarct are unlike what is found in human patients, with a smaller infarct core and a large penumbra. Photothrombosis does not produce a typical penumbra, as all vessels within the light beam will become occluded relatively equally (Labat-gest and Tomasi 2013). As for the cognitive deficits that are induced, the model is less able to target some of the deeper structures, although there are some papers describing deep brain tissues that can be affected, provided that the light has some means by which it can reach the target (e.g. fibre optics) (Kuroiwa et al. 2009). So far, however, there are no known photothrombotic models that target deep brain tissues that have a significant effect on

higher-order cognitive functions.

1.6.5 Endothelin-1

Endothelin-1, an endogenous 21 amino acid peptide that constricts blood vessels, has been used in many studies to help elicit ischemic conditions in animal models. This molecule is part of a family of endothelin molecules that work to constrict blood vessels by attaching to specific endothelin receptors found on smooth muscle cells which line the vessels (Yanagisawa et al. 1988). The most common of these, endothelin-1, can attach to G protein-coupled endothelin A and endothelin B receptors, and works to promote vessel constriction through the use of Ca^{2+} as a secondary messenger within muscle cells (Marsden et al. 1989). However, most of the action of endothelin-1 in the brain is done through endothelin A (ETA) receptors, as these are the dominant receptors expressed in vascular smooth muscle (Yu et al. 1995).

ET-1 can be directly injected onto large blood vessels, restricting the flow for a certain amount of time, mimicking an ischemic event (Ansari et al. 2013). As well, ET-1 can be injected directly into the parenchyma of the brain in order to cause ischemic damage through the vasoconstriction of smaller penetrating vessels (Hughes et al. 2003).

The vessels that have been primarily targeted with ET-1 include the MCA and the anterior cerebral artery (ACA), and each of these have demonstrated a varying degree of cognitive dysfunction (Sharkey et al. 1994; Ward et al. 1998; Lowrance et al. 2015). As well, Cordova et al. (2014) have demonstrated preliminary results that injections of ET-1 in the prefrontal cortex can have effects on executive function as

measured through an attentional set-shifting paradigm. As well, previous research in the Tasker laboratory has been able to use this model to produce deficits in motor function through the selected targeting of lesions in the motor strip (Livingston-Thomas et al. 2013; Livingston-Thomas et al. 2014).

Although the model does allow for targeted lesions in the rodent brain, there are some drawbacks to consider. The first of which is the induction of astrocytosis, the growth of new astrocytes, as well as the facilitation of axonal sprouting. However, these effects have only been noted within the spinal cord, and not from parenchymal injections directly into the cerebrum or cerebellum (Uesugi et al. 1996). As well, the endothelin model of stroke induction appears to be less effective in mice versus rats as the compound did not produce an ischemic lesion in four separate laboratory mouse strains that have been tested (Horie et al. 2008). The model has also been known to induce seizures, depending on the area of injection and age of the animal (Mátéffyová et al. 2006). Finally, the use of ET-1 to induce MCAo can produce a lesion of variable size depending on proximity of injection(Windle et al. 2006). Although there are some drawbacks to using endothelin-1 as previously mentioned, it provides a reliable and replicable method of inducing targeted focal ischemic lesions in the rodent brain. This is why it was selected as the method by which ischemic lesions would be induced within the work in this thesis.

1.7 TESTS OF HIGHER-ORDER COGNITIVE DYSFUNCTION IN EXPERIMENTAL RODENTS

When examining the cognitive capabilities of patients affected by stroke, there is often a verbal component within the exams, as well as tests requiring detailed verbal instructions for the patient to complete. For example, in the Montreal Cognitive Assessment (MoCA), a test that can be used to assess a patient's cognitive abilities (Nasreddine et al. 2005), the patient is asked to draw a clock in a particular fashion, to complete a two-item verbal abstraction task, and to complete a verbal fluency task. Unfortunately, directly assessing the cognitive capabilities of an animal by simply asking the animal to complete a particular task, or assessing the animal's verbal abstraction capabilities, is not possible. Therefore, our cognitive tasks need to be designed in such a way that the animal can complete a behavioural task requiring some level of cognitive capability without the use of verbal cues, designed to be analogous to existing behavioural tests used with humans. Reviewed here are a number of cognitive tasks used to assess particular aspects of rodent behaviour used in a variety of cognitive studies.

1.7.1 The Morris Water Maze (MWM) and Radial Arm Maze (RAM)

The MWM and RAM are some of the most widely utilized mazes for examining particular aspects of cognitive function; specifically they can be attuned to look for deficits in spatial memory and working memory, respectively (Davis et al. 1986; Hamm et al. 1996; Anderson et al. 2000). The MWM functions by the experimenter placing an animal in a large pool of opaque water with a hidden escape platform, which the rat

attempts to find in order to cease swimming. Over time, rats learn where the platform is located and the amount of time spent locating the platform decreases.

The RAM is also able to test for aspects of working memory by baiting certain arms of the maze with food rewards and measuring the amount of time it takes for the animals to locate the pellets. Counting the number of errors the animal makes indicates how proficient they are at the task (Olton and Samuelson 1976).

Both of these mazes have been separately used to assess aspects of cognitive decline in some models of stroke. For example, it has been shown that animals that have undergone an MCAo have increased times to find the platform, suggesting damage that has affected spatial memory (Dahlqvist et al. 2004). The RAM task has been used to assess cognitive function, specifically working memory, as a result of stroke. Animals that have undergone a four vessel occlusion fare worse in this task compared to control animals (Volpe et al. 1984).

The main purpose of these tests, particularly for the RAM, is for examining higher order cognitive function, specifically working memory. However, working memory is only one aspect of higher-order cognitive function, and these tests do not fully take into account aspects of inhibitory control, proper decision making, and attentional processing. For other higher-order cognitive functions to be adequately tested, we require a different set of mazes and tasks, some of which that are pertinent to this thesis will be described further.

1.7.2 Set Shifting Tasks

Although it is currently widely accepted that non-primates have functional equivalents to the human dorsolateral prefrontal cortex, at times it was believed that there were no homologous brain structures to this region (Preuss 1995). However, a seminal study done by Birrell and Brown (2000) demonstrated that damage to specific areas of the medial prefrontal cortex affected an animal's ability to switch from one now-irrelevant stimulus in its environment to a now-relevant stimulus, known as set-shifting.

Animal set shift tasks, which are used to assess how quickly an animal can shift its attention, are quite useful to test the higher-order cognitive functions of both non-human primates and rodents. Set shifting tasks function by training an animal to associate one feature of their environment with the receipt of a reward, to consider this choice as "correct", and then to switch the feature to a previously irrelevant environmental feature. This task is functionally analogous to what is performed in human subjects with the Wisconsin Card Sorting Test (WCST) (Birrell and Brown 2000). Other forms of set-shifting tasks have also been developed, relying on choice behaviour within modified t-mazes rather than different burrowing media or smells as found within Birrell's study (Stefani et al. 2003; Floresco, Ghods-Sharifi, et al. 2006). Each of these tests have demonstrated that affecting those regions of the brain most associated with higher-order cognitive functions, particularly the medial prefrontal cortex, affect set-shifting behaviour.

1.7.3 Temporal Object Recognition Tasks

One of the higher-order cognitive functions that can be affected by stroke is the understanding of events in the proper linear sequence in time, known as temporal order memory. This form of memory has broad implications for cognition and consciousness as a whole, as it is highly connected to episodic memory: memories about personal autobiographical events (St Jacques et al. 2008). Interestingly, it has been demonstrated that in humans temporal order memory can be affected without affecting object recognition within the tasks themselves (Kesner et al. 1994), providing evidence that this form of memory is truly about the linear nature of events. For testing a person's individual temporal order memory, we can employ a variety of tasks, including the examination of the serial position effect by placing a series of cards with words and pictures in the correct order (Shimamura et al. 1990; Schoo et al. 2014) or by getting participants to complete a series of tasks and to recall which events were performed in which sequence (Schmitter-Edgecombe and Seelye 2012).

Testing an animal's ability to place events in the correct sequence often relies on specific manipulations of object recognition tasks, and takes advantage of a rat's natural tendency to explore objects that are more novel to them (Ennaceur and Delacour 1988). For example, one version of a temporal object recognition task functions by placing an animal in an arena with two identical objects and allowing them to explore these objects for a certain amount of time, giving them a short break, and then allowing them to explore a completely different pair of identical objects. Finally, one of each of the two previously explored objects is placed within the arena, wherein the animal should spend

more time exploring the object seen during the first phase of the trial as compared to the second object, due to its greater degree of novelty (Hannesson et al. 2004; Warburton and Brown 2015).

1.7.4 Decision Making and Inhibitory Control Tasks

In human stroke patients, proper decision making and inhibitory control can be altered, which can have negative effects for these individuals (Bechara 2000; M. Hoffmann et al. 2010), and it has been noted that damage to the prefrontal cortex can cause both improper decision making and disinhibition (Manes et al. 2002).

Decision making refers to making the optimal choice amongst a different set of options, which can be tested in human patients through the use of gambling-like tasks including the Iowa Gambling Task (Brevers et al. 2013). This task requires individuals to pick cards from a series of decks, each of which will result in receiving "money" from the testers. Picking cards from certain decks will result in higher immediate rewards than other decks, however, picking cards from these decks will incur a delayed penalty for the participants, overall resulting in a net loss of money (Bechara et al. 1994). Picking cards from other decks presented will result in smaller gains of money, but will also result in considerably smaller penalties, leading to overall net gains. Although it is more advantageous to pick the decks with the lower reward, individuals with damage to parts of the prefrontal cortex pick the disadvantageous decks as compared to those with intact prefrontal cortices.

As with humans, rodents can also be tested in gambling-like tasks (Paine et al. 2013), which measure an animal's ability to make the optimal choice to obtain the highest amount of reward over a given span of time. Animals can also be tested in other cost-benefit decision making tasks, which typically offer the choice between two sets of options: a higher-valued reward and a lower-valued reward. All things being equal, the higher-valued reward would be hypothesized to be chosen more frequently. However, there is a cost to choosing the higher-valued reward, typically being the amount of time required to wait for the higher-reward. In this task, animals with an intact prefrontal cortex will more often choose to wait for the larger reward as compared to lesioned animals (Rudebeck et al. 2006).

Effort based decision making is similar in nature to cost-benefit decision making, the cost in this case being the amount of effort required to obtain the reward. This behaviour has been tested by, again, employing a t-maze configuration, with one arm providing easy access to a low food reward, and the other arm having a physical obstacle to overcome to obtain a higher food reward (Walton et al. 2002). Interestingly, the areas of the brain primarily responsible for this particular task are thought to be slightly different as compared to other decision making or inhibitory control tasks (Walton et al. 2003), again suggesting that the rodent prefrontal cortex can be further subdivided into regions controlling particular actions, which will now be explored further.

1.8 EXECUTIVE FUNCTION AND THE PREFRONTAL CORTEX

The exact definition of what are, or are not, executive functions has been discussed thoroughly in the literature for a number of years. Essentially, the term refers to a set of top-down controls of mental processes determining the decision making of a particular organism (Diamond 2013). Many of the previously discussed higher-order cognitive functions fit into this realm of executive functions, including attentional processing, working memory, planning, decision making, and the temporal integration of information (Fuster 2005).

The brain region thought to be most in control of executive functions in mammals is the prefrontal cortex (PFC) (Fuster 2005), and, as a result, this region has been extensively studied through various lesion studies in attempt to determine which particular behaviours are controlled by which regions of the PFC.

1.8.1 Review of lesion studies with the prefrontal cortex and its effects on cognition

The prefrontal cortex is seen as the seat of executive function, which directs and oversees the behaviour of the organism (Miller and Cohen 2001). In previous years there has been dispute as to whether rats and other rodent species have a true prefrontal cortex, with regions similar to what are found in non-human primates and humans (Preuss 1995). Although the range of behaviours exhibited by rats as compared to humans is limited, behavioural and cytoarchitectural experiments provide reasonable

evidence to conclude that rats do have a prefrontal cortex comparable to our own, controlling similar functions (Uylings et al. 2003).

Detailed below, are examples of studies examining the effects of lesions within this brain region and its effects on cognition in rodent models, non-human primates, and human patients.

1.8.1.1 Human Studies

Although studies examining brain injury of the prefrontal cortex in humans are typically case studies and therefore retrospective in nature, there is still much to be learned from them. Perhaps the best known historical case study regarding prefrontal cortical damage was the case of Phineas Gage, who suffered an industrial accident leading to a railroad spike penetrating and damaging parts of Mr. Gage's prefrontal cortex (Harlow 1999)(Reprint). Although Mr. Gage physically recovered from his injuries and survived a number of years post-trauma, his personality had drastically changed becoming unreliable at work, fitful, impatient, and he was described as "[a] child in his intellectual abilities" (Harlow 1868).

Over the past number of years there have been well-documented cases and studies regarding damage to specific areas of the prefrontal cortex correlating well with deficits in particular cognitive functions and certain irregular behaviours. These behaviours can include some of those described by Dr. Harlow with regards to Phineas Gage, as well as others that have been well documented. For example, patients experiencing damage to the PFC have difficulties understanding the consequences of

their future actions as assessed through decision-making tasks mimicking real life choices (Bechara et al. 1994).

The human prefrontal cortex, much like the prefrontal cortex of other animals, can be further subdivided anatomically (Siddiqui et al. 2008). Further sub-categorization of these areas has determined that the dorsolateral prefrontal cortex is activated during attentional set-shifting, and that damage to the dorsolateral prefrontal cortex (dlPFC) is associated with deficits in function in the attentional set shifting task (Milner 1963; Konishi et al. 1998). Other processes that appear to be affected by damage to this region include proper decision making, as assessed through the Iowa Gambling Task (Fellows and Farah 2005), as described previously.

Another further subdivision of the prefrontal cortex includes the ventromedial portion (vmPFC), which controls areas related to reversal learning and attentional processing, similar to that seen in non-human primates (Fellows and Farah 2003). Ventromedial lesions also appear to affect moral judgements (Ciaramelli et al. 2007), causing patients to make more "utilitarian" moral decisions.

From many of these studies, it is clear that the prefrontal cortex plays a critical role in executive functions and higher order cognition. Although other areas can play a role in some behaviours associated with executive function, in both humans and other animals (Stuss and Alexander 2000; Jahanshahi et al. 2002; Balleine et al. 2007), none have as profound or as well documented a role as the PFC.

1.8.1.2 Non-Human Primate Models

The effects of prefrontal cortical lesions has also been extensively studied in non-human primates, as these animals are most closely related to humans and have similar anatomical features within their prefrontal cortex (Petrides and Pandya 1999; Semendeferi et al. 2002; Ongür et al. 2003).

Related to their similar anatomical features, there is considerable evidence that particular regions of the non-human primate prefrontal cortex relate to particular behaviours controlled by the human prefrontal cortex. For example, the study of prefrontal cortical lesions in macaque monkeys has indicated that attentional processing can be damaged by lesions to the lateral prefrontal cortex (Rossi et al. 2007), similar to what is reported in human findings examining functional locations (Asplund et al. 2010). As well, rhesus macaques have also had reported difficulty with attentional set shifting tasks analogous to impairments in the WCST following damage to the prefrontal cortex in humans (Moore et al. 2009).

1.8.1.3 Rodent Modelling

Some of the first studies examining the effects of frontal lesions on the behaviour of the rat were done by Bourke et al (1954), in which they examined the effects of lesions in white rats, finding that these lesions caused changes to habit behaviour in these animals. Other earlier studies regarding parts of the frontal cortex

used object recognition, which can be affected by frontal damage (Becker and Olton 1980)

Further testing with more complicated tasks revealed that not only could these animals perform these more complicated tasks, but that lesions within the prefrontal cortex caused deficits in these tasks as compared to control animals. For example, reversal learning, thought to be dependent on certain subsections of the prefrontal cortex, was impaired by lesions to the medial prefrontal cortex (de Bruin et al. 1994). Further studies indicated that rats required a particular portion of the prefrontal cortex to perform set-shifting, as explored by Birrell and Brown (2000). Since then, other methodologies for testing and comparing the cognitive capabilities of the rodent's prefrontal cortex to the human's have been established, including other tests of attention, choice behaviour, and decision making.

1.9 REVIEW

The previous sections of this review have given a detailed background into the disease of stroke, the current deficits those that suffer from a stroke may face, and the current state of the literature regarding the modelling of stroke. This disease is debilitating and to date few regulated and implemented treatment options for those who suffer from profound cognitive deficits exist. Despite this, a very small subset of research concentrates on the alleviation of cognitive deficits post-stroke, and there are few models that concentrate specifically on "higher-order" or executive function deficits.

The purpose of this body of work conducted and reported in this thesis is to develop, test, and examine a new animal model of stroke that is intended to cause significant higher-order cognitive deficits in rats. This body of research and development was done with the goal that this new model can be utilized to test behavioural or pharmacological therapies to treat the cognitive symptoms associated with stroke.

1.9.1 Hypothesis

The hypothesis for this thesis is that injections of the vasoconstrictor, endothelin-1, into particular regions of the prefrontal cortex, will cause ischemic lesions in this area, as well as measureable cognitive deficits in higher-order cognitive functions. These will be measured through the use of particular histological experiments and behavioural assessments.

1.9.2 Objectives of the Thesis

The use of endothelin-1 to induce ischemic lesions in the prefrontal cortex, as a model of stroke, will be evaluated through the following three objectives:

1. To refine and evaluate the effects of endothelin-1 injections into the prefrontal cortex on brain lesion size, and changes in behaviour in the rat.

2. To measure the effects of refined, focal ischemic lesions within the medial prefrontal cortex on aspects of set-shifting and temporal order memory, both of which are higher-order cognitive functions, in the rat.
3. To further evaluate the model using endothelin-1 to create focal lesions in the orbital prefrontal cortex versus lesions in the medial prefrontal cortex for the purposes of assessing regional effects.

Chapter 2 of this thesis will explore the first objective, which was to establish the stroke model through testing two separate ET-1 injection protocols. Once an ET-1 injection protocol was selected due to its targeted focal ischemic lesion to the rodent mPFC, preliminary experiments were conducted to understand the behavioural effects of this stroke lesion. In Chapter 3 the second objective, which is to test higher-order cognitive functions in the rat, was explored using the animal model established from Chapter 2. Tests of higher-order cognitive function, specifically set-shifting and temporal object recognition, were employed to understand how stroke lesions in the mPFC would affect attention and temporal memory. Chapter 4 will examine the final objective, which was to examine two separate ET-1 models affecting the mPFC and oPFC and to determine whether these models had effects on inhibitory control and ultrasonic vocalizations. Finally, Chapter 5 will provide a general overview of the thesis and outline future experiments to further the knowledge obtained by the experiments performed in chapters two through four.

CHAPTER 2

PREPARATORY EVALUATION AND VALIDATION OF MODELS OF POST-ISCHEMIC COGNITIVE DYSFUNCTION

SUMMARY

Modelling ischemic strokes that cause damage specific to cognitive functions is an area of research which has been overshadowed by other animal models concentrating on the recapitulation of the motor dysfunctions of this disease. The majority of research examining dysfunction following stroke makes use of the middle cerebral artery occlusion model (MCAo), which has little documented evidence of higher order cognitive deficits. Therefore, to properly model higher-order cognitive dysfunction post-stroke, new models are required. The experiments described in this chapter had two aims: (1) to evaluate the cellular damage caused by the injection of endothelin-1 (ET-1), a potent vasoconstrictor, into the medial prefrontal cortex, and (2) to evaluate a model of ET-1 damage for evidence of cognitive dysfunction using two separate behavioural tasks. The first experiments demonstrated that the vasoconstrictor ET-1 can cause damage to the prefrontal cortex, and that a model consisting of a pair of bilateral injections was suitable for the purposes of subsequent experimental procedures. The second set of experiments used this two-injection model, and two different behavioural tests–the elevated plus maze (EPM) and the temporal object recognition task (TOR) –to test the outcomes of this surgical procedure. The animals displayed increased levels of anxiety as a result of this surgical procedure relative to the control group, as measured through the EPM. The TOR task, employed to test an animal's temporal order memory, failed to properly measure this outcome in either the sham or stroke group, which would indicate that this task requires further testing modifications before future use.

2.1 INTRODUCTION

Stroke is one of the main forms of death and disability worldwide, with an estimated 5.7 million deaths worldwide and 16 million new strokes that occur per year (Strong et al. 2007). The only major pharmaceutical product given to help prevent damage post-stroke, recombinant tissue plasminogen activator (r-TPA), is administered to ischemic stroke patients within 3 - 6 hours of stroke onset and has been shown to cause considerable improvements in functional outcome (Lubeck et al. 2016). Although this drug is effective at preventing tissue damage post-stroke, it can only be administered to patients under specific circumstances, and it has been estimated that only 7% of ischemic stroke patients receive r-TPA post-injury (Schwamm et al. 2013).

Current preclinical stroke research has been very successful at replicating many of the signs of upper limb motor dysfunction following stroke in animals, which has led to a better understanding of the cellular and molecular mechanisms underlying neuroplasticity post-stroke (Kleim et al. 2007). Unfortunately, there is a lack of animal models of stroke that cause complex cognitive deficits, despite the fact that approximately one third of stroke survivors suffer from cognitive-dysfunction post-injury (Tatemichi et al. 1994; Patel et al. 2003). To help remedy this situation, new models of cognitive dysfunction post-stroke are needed.

Endothelin is a potent vasoconstrictor initially discovered in 1988 in cell culture (Yanagisawa et al. 1988). Further investigation revealed that endothelin-1 (ET-1), the most biologically prominent of the four discovered isoforms of endothelin, could be used directly to occlude blood vessels *in vivo* (Sharkey and Butcher 1995; Luscher and

Barton 2000). Since its discovery and subsequent characterisation, this compound has been used in a host of vascular impairment models in order to study the effects of stroke on behaviour *in vivo* (Thiyagarajan and Sharma 2004; Faraji et al. 2009; Livingston-Thomas et al. 2014). Until recently, however, use of this drug to cause potential cognitive dysfunction through targeted injections into the brain had not been described.

The purposes of the experiments described in this chapter were two-fold. First, initial experiments were performed to assess the relative size and dimensions of damage caused by endothelin-1 injections to the prefrontal cortex in two sets of injection protocols. The second purpose of the experiments was to assess the behavioural effects of one of the injection protocols. The two tests employed in the experiment were the elevated plus maze to assess for changes to anxiety-related behaviour, and a temporal object recognition test used to assess an animal's ability to differentiate different time intervals, as previous experiments have demonstrated a relationship between frontal lobe damage and dysfunctions in temporal order memory.

The hypothesis of these initial experiments was that injections of ET-1 would cause measurable ischemic lesions in the prefrontal cortex, and that these lesions would have measureable (statistically significant) effects on behaviour and cognitive function.

2.2 METHODS

2.2.1 Experiment 1: Development of an Injection Protocol

Two separate protocols were assessed: one for two sets of bilateral injections of ET-1 to the PFC (n = 6) and one for one set of bilateral injections of ET-1 to the PFC (n = 5). With the exception of the number of injections performed, the surgical protocols were identical.

The surgical protocols for the injections were performed as follows. Adult Sprague Dawley rats (weights 400 - 600 g) were used, and each rat was anaesthetised by being placed into an induction box prefilled with 3.5% isoflurane (PPC, Richmond Hill, Canada) in oxygen, and was left in the box for 8 minutes. The anaesthesia was maintained throughout the surgery with a 2-3% isoflurane mixture. After 8 minutes, animals were mounted on a stereotaxic frame (Kopf Instruments, Tujunga, USA), and the heads were shaved. Once shaved, topical Xylocaine (AstraZeneca, Mississauga, Canada) was applied to the shaved area and left for 5 minutes, following which a 2 cm midline incision was made down the top of the cranium. This incision was held open by 4 clamps.

Depending on which surgical injection procedure was performed, the coordinates differed for each rat. Two to four small burr holes were drilled into the cranium above the coordinates for drug injection. A 26-gauge 10 µl syringe (Hamilton, Reno, USA) was lowered into each of the injection sites using coordinates as listed in the following tables. The exact coordinates for each of these injection protocols are given in Tables 2.1 and 2.2.

Table 2.1 Surgical injection coordinates for a 4 injection protocol. A/P = anterior/posterior, M/L = medial/lateral, D/V = dorsal/ventral, with distances in mm relative to bregma. n = 6.

	A/P (mm)	M/L (mm)	D/V (mm)	Volume (µl)
Injection 1	+3.5	-0.7	-4.5	1
Injection 2	+3.5	+0.7	-4.5	1
Injection 3	+2.5	-0.7	-4.5	1
Injection 4	+2.5	+0.7	-4.5	1

Table 2.2 Surgical injection coordinates for a 2 injection protocol. A/P = anterior/posterior, M/L = medial/lateral, D/V = dorsal/ventral, with distances in mm relative to bregma. n = 5.

	A/P (mm)	M/L (mm)	D/V (mm)	Volume (µl)
Injection 1	+3.0	-0.7	-4.5	1
Injection 2	+3.0	+0.7	-4.5	1

In each case the injection needle was left in place for 1 minute prior to ET-1 injection. After one minute, 1 µl of ET-1 (400 pmol) dissolved in artificial cerebral spinal fluid (aCSF, see appendix A for composition) was injected at each injection point into the cortex at a rate of 0.5 µl/minute for a total of two minutes.

Once injected, the needle was left in place for 4 minutes to allow for drug dispersal at the injection site, and then the needle was slowly retracted from the brain. The same procedure was repeated for each injection point. After the ET-1 injections, the incision site was sutured and topical Xylocaine was reapplied. At the conclusion of the surgery each animal was given a 2.0 mg/kg subcutaneous injection of butorphanol tartrate. Over the course of the recoveries, four of the animals either died or had to be euthanized post-surgery, of which one was from the two injection group and three were from the four-injection group. Therefore, the final group numbers analyzed for each group were n = 3 for the four-injection group and n = 4 for the two injection group.

2.2.1.1 Histology

On post-surgery day 4 (PSD4), the surviving rats were anaesthetized with isoflurane and euthanized by decapitation. Brains were removed and immersion fixed in 10% formalin. Brain tissue was sectioned into 100µm coronal segments throughout the anterior section of the brain using a vibratome (Ted Pella, Redding, USA), mounted on Surgipath X-tra glass slides (Leica Biosystems, Richmond, USA) and subsequently stained with cresyl violet (0.1 % w/v). Images were prepared using a Nikon D3100 digital camera and a light box. Infarct quantification was performed by taking each

digital image and tracing the area of tissue displaying a lack of, or abnormal, cresyl violet staining or altered cytological architecture. Tracing was performed using ImageJ software, version 1.45s (ImageJ, National Institutes of Health, USA). Once each area had been traced, the volume of infarct damage was quantified by summing each individually calculated area and multiplying the area by the distance between each section (100 µm).

2.2.1.2 Statistical Analysis

Statistical analyses were performed using Graph Pad Prism, Version 5.00 (Graph Pad, La Jolla, USA). In this first experiment, the two sets of injection protocol lesion sizes were assessed by a two-way ANOVA. In the analysis, statistical significance was considered at $p \leq 0.05$.

2.2.2 EXPERIMENT 2: BEHAVIOURAL TESTING

2.2.2.1 Experimental Animals

All procedures were conducted in accordance with the guidelines of the Canadian Council for Animal Care and were approved in advance by the University of Prince Edward Island Animal Care Committee, protocol number 12-035. Adult male Sprague-Dawley rats (N = 21, 126-150g on arrival) were purchased from Charles River Laboratories (Montreal, Canada) and singly housed on a 12 h light/dark cycle (lights on

at 06:00 and off at 18:00) with food (Purina rat chow) and water available *ad libitum* until training and testing. Upon arrival, the animals were handled by the experimenter for 5 minutes each day for three days during an acclimation period. All surgeries, behavioural training, and behavioural testing occurred during the light phase of the light cycle.

2.2.2.2 Surgical Procedures

The two-injection model, as described in section 2.2.1, was performed on the 21 male Sprague-Dawley rats, with 11 rats in the ET-1 injected group, and 10 rats in the sham injection group receiving vehicle control (1 µl of aCSF per injection point). Post-surgery, three animals were euthanized for humane reasons; two from the ET-1 group, and one from the sham group. Therefore, the final group numbers were n = 9 for each group. For exact details regarding the methodology of the surgery, refer to section 2.2.1.

2.2.2.3 Behavioural Testing Protocols

Testing for the elevated plus maze (EPM) was completed during the light phase of the light/dark cycle. Testing consisted of placing the animal in an elevated plus maze platform (elevated 83 cm from the floor of the room), constructed of welded metal with the floor of the maze covered with adhesive liner. Each of the four arms of the maze had dimensions of 112 (length) × 10 (width) cm. Two of the arms had no walls and were exposed (open arms), and the other two arms had metal walls which were 28 cm high

(closed arms). Once the animals were placed in the centre platform of the maze, the timer was started and animals had 5 minutes to explore the maze. Behaviour was video-recorded using a Logitech webcam (Logitech, Romanel-sur-Morges, Switzerland) suspended over the centre of the maze, connected to a laptop, using ANY-Maze Software version 4.76 (Stoelting Co., USA). The variables tested during the experiment were the amount of time spent in the closed arms, the amount of time spent immobile, the amount of distance moved throughout the trial, and the number of rearing motions made during testing. Animals were tested three times over the course of the experiment; pre-surgery, on PSD8, and on PSD29.

The testing procedure for the temporal object recognition task was modified from Hannesson et al. (2004). The testing area consisted of a round, empty, fibreglass container (148.6 cm in diameter; walls 63.5 cm in height). To aid in colour contrast for video recording, the floor of the maze was covered in brown adhesive shelf liner. On PSD5 and PSD6 each animal was acclimated separately to the maze for 10 minutes. Testing began on PSD7 and consisted of placing the animal in the maze with a pair of identical objects positioned equidistant from the centre of the maze and the maze walls. The rat was allowed to explore for 4 minutes before being returned to its home cage. After a delay of 60 minutes, the animal was placed back in the maze with a new pair of identical objects for a further 4 minutes. After an additional delay of 180 minutes, the animal was returned to the maze with one object from each of the two previous trials for 3 minutes. The time spent exploring each of the objects was recorded. Exploration of an object was defined as the animal's head being within at least two centimetres of the

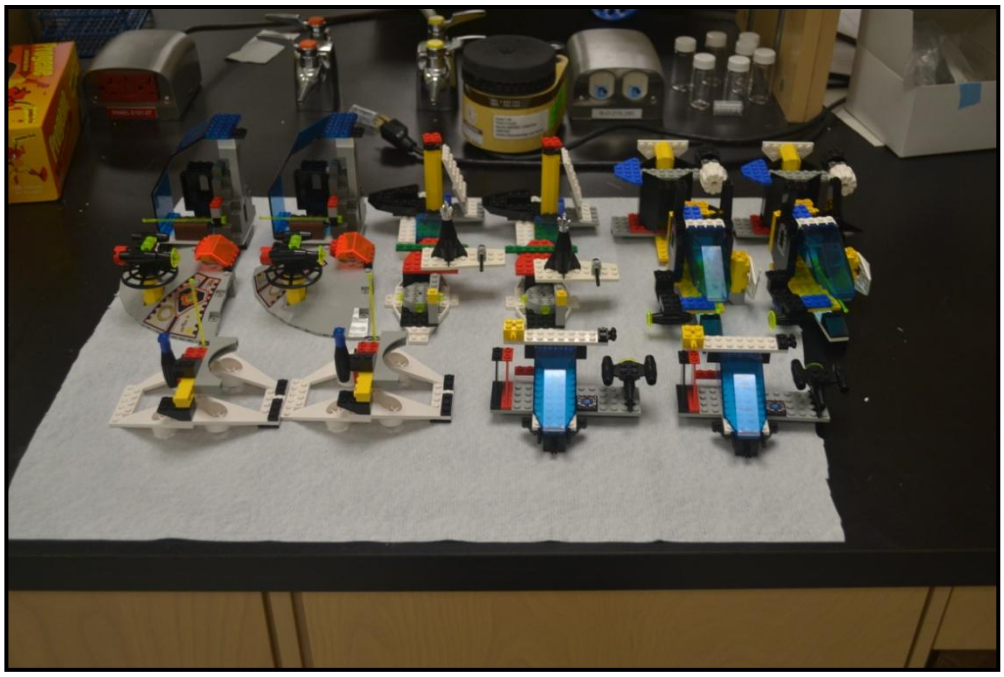


Figure 2.1 Objects used for TOR testing. Each set of objects was used once during the four testing periods.

object's surface, and scoring was done using AnyMaze software (Stoelting, Wood Dale, USA). TOR testing took place on PSD7, PSD14, PSD21, and PSD28. Two sets of objects were used during this experiment, with a different set of plastic Lego objects tested each week. Examples of the objects used are given in Figure 2.1.

2.2.2.4 Histology and Infarct Quantification

On PSD29, the experimental animals were anaesthetized with isoflurane and euthanized by decapitation. Brains were removed and immersion fixed in 10% formalin. Brain tissues were sectioned, mounted, stained and infarcts quantified as described previously in Section 2.2.2.1

2.2.2.5 Statistical Analysis

Statistical analyses were performed using Graph Pad Prism, Version 5.00 (Graph Pad, La Jolla, USA). The outcomes of the behavioural tests employed were assessed via two-way repeated measures ANOVAs comparing the day of testing with the surgery group. Post-hoc comparisons were made using Bonferroni post-test, one way ANOVAs, or t-tests where appropriate. In all analyses, statistical significance was considered at $p \leq 0.05$.

2.3 RESULTS

2.3.1. Experiment 1 - Histology

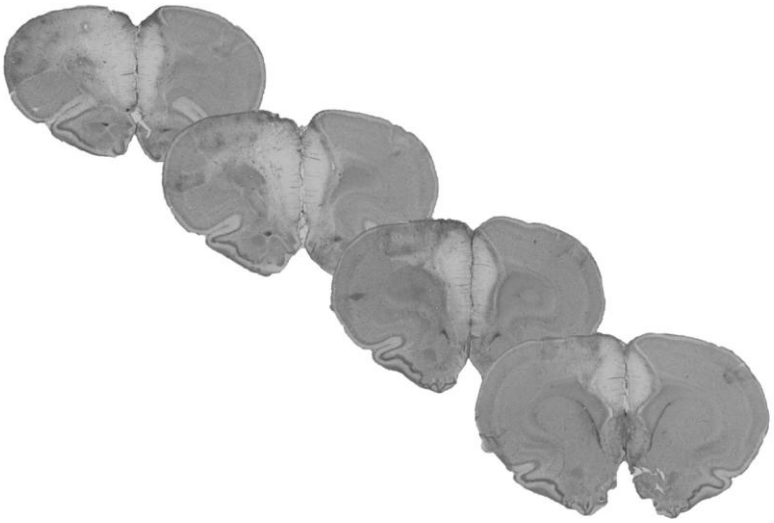
Histological analysis of the infarct produced in the two different surgical protocols revealed that both injection protocols damaged the area of the mPFC as anticipated (Figures 2.2 A and B). Comparison of the two and four injection protocols using a two-way ANOVA of protocol versus infarct area at each 300 µm section, revealed a statistically significant difference between the two-injection and four-injection models ($F_{1,19} = 22.0$, $p < 0.01$). Figure 2.3 displays each of the estimated areas of damage at 300 µm intervals. Although there was no statistical difference in the number of mortailities between each of the surgical injection grouns, becauseof the more focused damage to the mPFC and overall fewer animal mortailities, the two injection model was used for future injection protocols.

2.3.2. Experiment 2

2.3.2.1 Histology

Figures 2.4 A and B demonstrate the extent of damage produced following bilateral endothelin-1 injections into the mPFC as examined 29 days post stroke surgery. Figure 2.4 A gives an overview of the damage, with four coronal sections through the infarcted area. Figure 2.4 B demonstrates an overview of the estimated volume of.

A.



B.

Figure 2.2. Cresyl violet- stained brain tissue following the two- and four-injection ET-1 models. Damaged tissue is represented by lower stain uptake, as well as altered cytoarchitecture. (A) Representative sections following the 4 injection mPFC protocol and (B) representative sections following the 2 injection mPFC protocol.

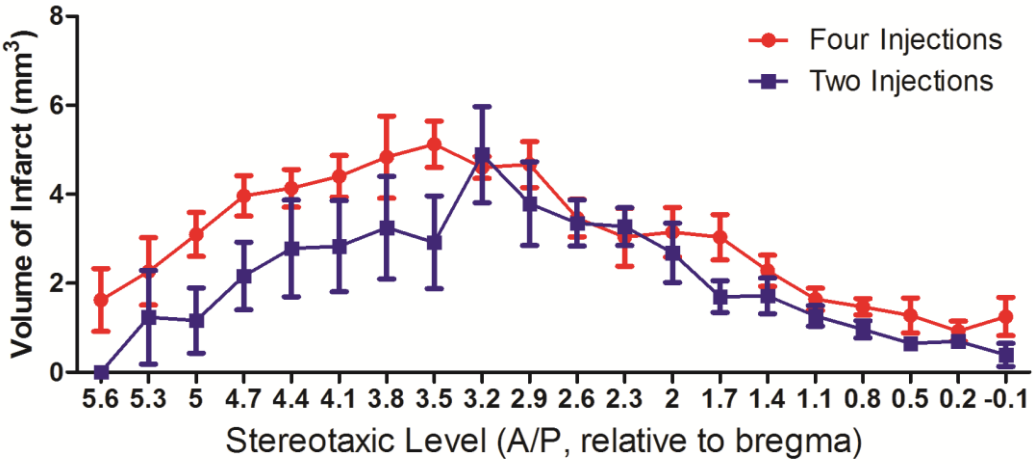


Figure 2.3. Line graphs representing the approximate amount of damaged tissue as a result of each ET-1 surgical injection protocol. The approximate amount of area damaged was calculated every 300 µm from the anterior of the brain to approximately bregma. For the four-injection group, n = 3, and for the two-injection group, n = 4.

A.

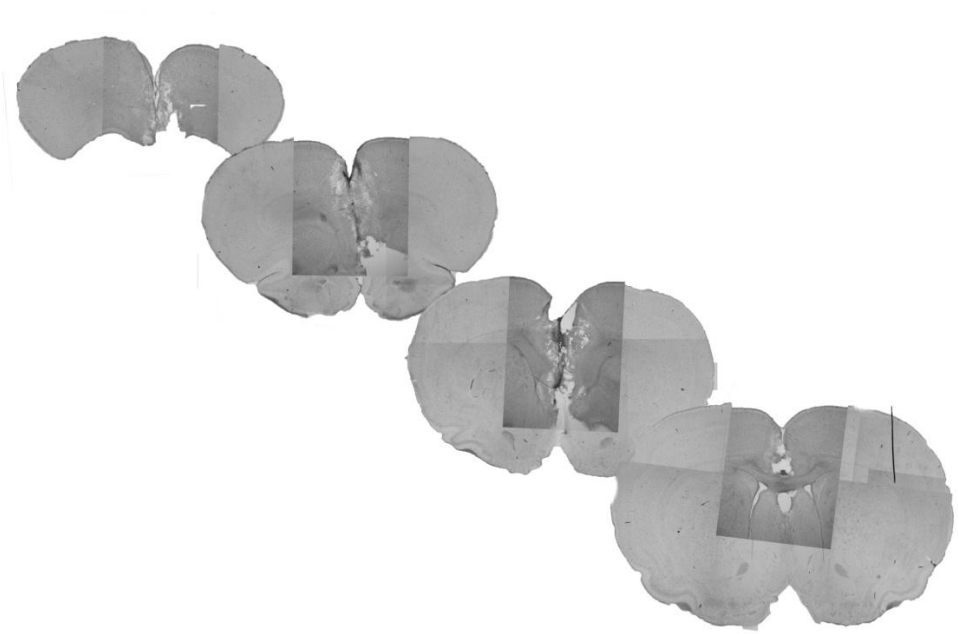


Figure 2.4A A representative example of the ischemic insult found within the prefrontal cortex within the experiments described in section 2.2.2., displayed through successive coronal sections of the area of interest.

B.

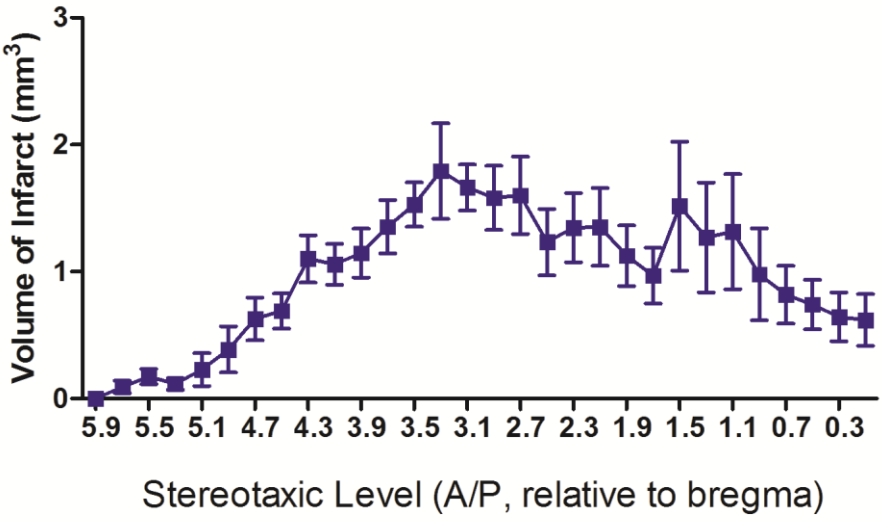


Figure 2.4 B Line graph representing the relative volume of damage at different stereotaxic levels in the ET-1 group (n = 9) within the experiments described in section 2.2.2.

damage every 200 µm, demonstrating the largest amount of damage occurred between +3.3 mm and +2.7 mm anterior to bregma, coinciding with the bilateral injections placed at +3.0 mm anterior to bregma. Total size of the infarct was 27.98 ± 3.917 (Mean ± SEM).

2.3.2.2 Elevated Plus Maze

Animals were tested on the EPM pre-surgery, on PSD8, and again on PSD29. Analyses of the EPM data suggest there were differences between the sham and stroke groups, as well as differences within the stroke group depending on when the animals were tested. Figure 2.5 gives an initial overview of the differences in time spent in the closed arms of the EPM. A two-way ANOVA revealed an interactive effect between the day tested and the surgery group ($F_{(2,16)} = 3.78$, $p < 0.05$), so subsequent analyses looked at each of these factors separately. As assessed by a one-way ANOVA between testing days there were no statistically significant differences within surgery groups (sham: $F_{(2,8)} = 3.56$, $p > 0.05$; stroke: $F_{(2,8)} = 1.25$, $p > 0.05$). However, as assessed by an unpaired t-test there was a significant difference between surgical groups on PSD29, with animals in the stroke group spending more time in the closed arms of the maze than the open arms ($t_{(16)} = 2.340$, $p < 0.05$). There was no significant difference between sham or stroke groups pre-surgery or on PSD8.

In terms of the amount of distance moved in the maze during the testing period, a two-way repeated measures ANOVA comparing the effects of the surgical procedure and the day of testing revealed statistically significant differences between testing days ($F_{(2,16)} = 6.08$, $p < 0.01$) (Figure 2.6A). Bonferroni post-hoc analysis revealed that for

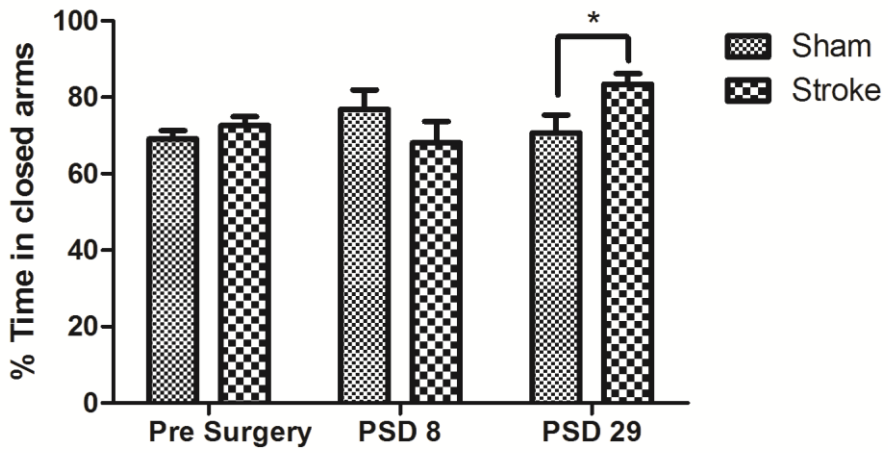


Figure 2.5 Percent time spent in the closed arms of the EPM pre- and post-surgery. Two-way repeated measures ANOVA revealed an interactive effect between the day tested (pre-surgery, PSD8, and PSD29) and the surgical group (sham or stroke, n = 9 for each group). Further post-hoc testing revealed there was a difference between sham and stroke animals on PSD29, indicating that stroke animals spent more time in the closed arms relative to their sham counterparts. Bars indicate mean ± SEM of time spent in closed arms.

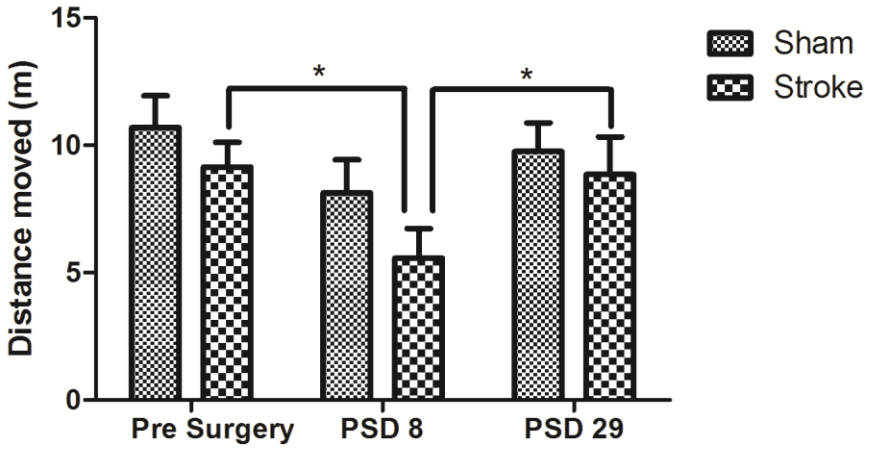


Figure 2.6A. Distance moved in the EPM pre- and post-surgery. A two-way repeated measures ANOVA indicated a time effect, wherein stroke animals performed differently as compared to their pre-surgery and post-surgery day 29 values. There was no significant effect between surgical groups (n = 9 for both groups).

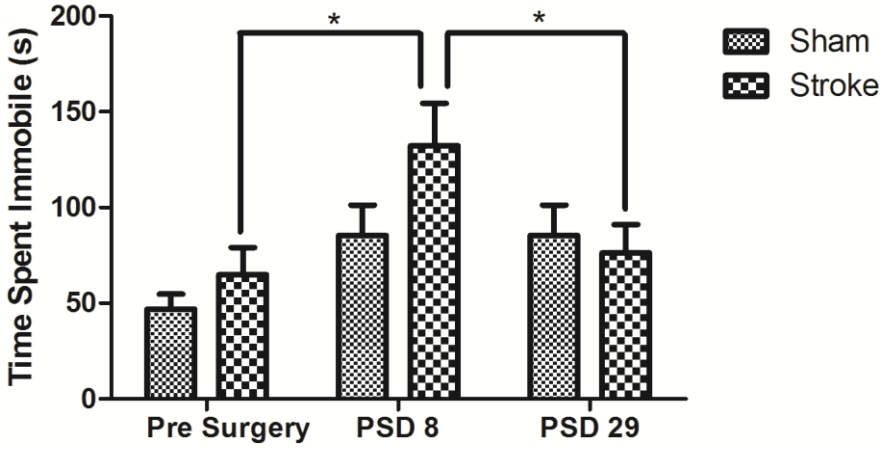


Figure 2.6B Time spent immobile in the EPM pre- and post-surgery. Similar to distance moved, a two-way repeated measures ANOVA indicated a time effect, wherein stroke animals performed differently as compared to their pre-surgery and post-surgery day 29 values. No significant difference was detected between sham and stroke animals (n = 9 for both groups).

stroke animals, the amount of distance moved on PSD8 was lower than pre-surgery and PSD29 (Pre-surgery vs PSD8 $t_{(8)} = 2.72$, $p < 0.05$) (PSD29 vs PSD8 $t_{(8)} = 2.49$ $p < 0.05$). No statistical differences were found between test days for the sham-treated group. A similar result was found for the amount of time the animals spent immobile in the maze.A two-way repeated measures ANOVA comparing the effects of the surgical procedure and the day of testing found a significant effect due to the testing day ($F_{(2,16)} = 7.86$, $p < 0.01$). As well, upon post-hoc analysis this effect was only seen in stroke animals comparing between pre-surgery and PSD8 ($t_{(8)} = 3.56$, $p < 0.05$), as well as between PSD29 and PSD8 ($t_{(8)} = 2.96$, $p < 0.05$). No statistically-significant effects were seen in post-hoc analyses between days in the sham group. With regard to rearing behaviour while in the maze, a two-way repeated measures analysis revealed statistically significant differences between sham and stroke group behaviour ($F_{(1,16)} = 7.55$, $p < 0.05$) (Figure 2.7), no differences between days were found ($F_{(2,16)} = .779$, $p > 0.05$). Further Bonferroni post-hoc analysis revealed that these differences were only significant between groups on PSD8 ($t_{(8)} = 3.02$, $p < 0.05$).

2.3.2.3 Temporal Object Recognition

Table 2.3 gives an overview of the results of the temporal object recognition experiments. For each day tested (PSD7, PSD14, PSD21, and PSD28), a two-way ANOVA was performed examining the time spent with each object within and between the surgical groups. On PSD7, there was no statistical difference between time spent with either the first or second object ($F_{(1,16)} = .010$, $p > 0.05$), or between the sham and stroke groups ($F_{(1,16)} = 3.02$, $p > 0.05$). On PSD14, there was a significant interactive effect between the surgical groups and object preference ($F_{(1,16)} = 5.25$, $p < 0.05$). Further

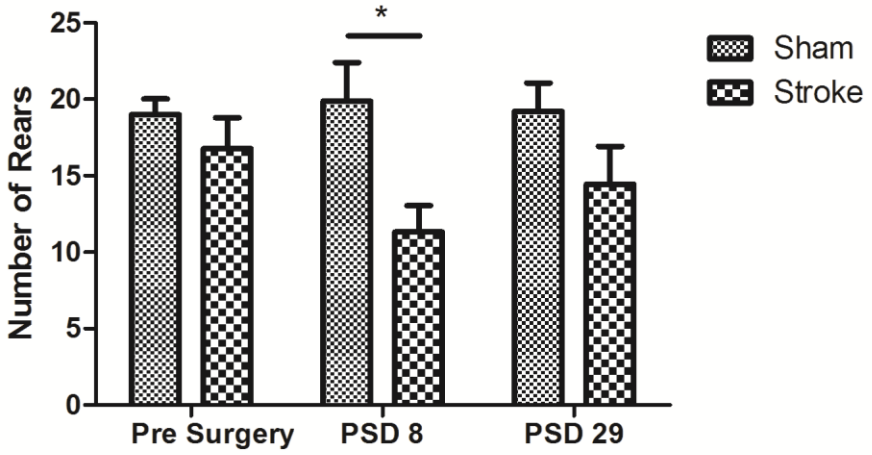


Figure 2.7 Number of rearing actions performed in the EPM. A two-way repeated measures ANOVA revealed a significant difference between sham (n = 9) and stroke animals (n = 9), and further post-hoc analysis revealed a significant difference between sham and stroke animals on PSD8, with animals in the stroke group performing less rearing motions than sham animals.

Table 2.3 Average time interacting with objects in TOR maze. For each day tested, a two-way ANOVA was performed examining the effects of surgery on the time spent interacting with each of the two objects. There was no statistically-significant difference between either time spent with the objects or between surgery groups on PSD7. On PSD14, an interactive effect was found between time spent with the objects and surgical group (n = 9 for both groups). Further testing revealed a statistically-significant difference between the examination time between the sham and stroke group regarding the amount of time spent with the object presented second ($P < 0.05$). For both PSD21 and PSD28, no statistical difference between either the time spent with objects nor the surgery group was found. Data are presented as Mean ± SEM.

	Sham		**Stroke**	
Testing Day	First Object	Second Object	First Object	Second Object
PSD 7	16.5 ± 2.52	14.6 ± 2.99	8.41 ± 2.97	11.3 ± 4.34
PSD 14	20.3 ± 3.38	**18.8 ± 2.98***	38.79 ± 12.4	**7.57 ± 3.67***
PSD 21	11.4 ± 1.76	11.6 ± 1.30	11.9 ± 4.21	21.4 ± 5.59
PSD 28	16.6 ± 3.28	11.4 ± 3.63	13.8 ± 4.43	9.59 ± 2.48

investigation revealed a significant difference in the amount of time spent exploring the second object ($t_{(8)} = 2.42$, $p < 0.05$). No other statistically significant differences were found on PSD14. As well, two-way ANOVA analyses revealed no statistically significant differences between sham and stroke groups, or between the amount of time spent exploring each set of objects for neither PSD21 nor PSD29.

Table 2.4 gives an overview of the distance moved in the maze. Through a two-way repeated measures ANOVA it was revealed that there was a statistically significant difference between sham and stroke groups ($F_{(3,16)} = 11.0$, $p < 0.01$) with stroke group rats appearing to move less in the maze. Further post-hoc testing revealed that this effect was statistically-significant only for PSD14 ($t_{(8)} = 3.72$, $p < 0.01$)

2.4 DISCUSSION

To date, much of the current research into cognitive dysfunction post-stroke has examined deficits in working memory, spatial memory, and learning ability (Jiwa et al. 2010). This may be because one of the most prominent areas of the brain that is damaged following global hypoperfusion and MCA occlusion is the hippocampus, an area intricately involved with spatial and working memory (Butler et al. 2002; Hartman et al. 2005). Although the hippocampus does play a role in some executive functions, it is the prefrontal cortex that is most involved with higher-order cognitive functions including goal directed behaviour and proper decision making (Miller and Cohen 2001). It is for this reason that the experiments in this chapter aimed to establish reliable lesions to certain sections of the prefrontal cortex and to assess behaviours associated primarily

Table 2.4 Average distance moved in TOR maze. A two-way repeated measures ANOVA revealed a significant effect ($P < 0.05$) of surgery on the distance moved in the TOR maze. Post-hoc Bonferroni testing between groups on each day indicated that on PSD 14 sham animals (n = 9) moved greater distances over the three minutes in the testing apparatus as compared to stroke animals (n = 9). Data are presented as Mean ± SEM

	Distance moved (m)	
Testing Day	**Sham**	**Stroke**
PSD 7	11.2 ± 1.13	6.49 ± 1.92
PSD 14	13.4 ± 1.58	5.28 ± 1.40 *
PSD 21	11.1 ± 1.26	6.02 ± 1.15
PSD 28	12.7 ± 1.82	8.45 ± 1.77

with this region.

The first set of experiments was performed in order to understand the effects of a four injection model of ET-1 versus a two injection model. What was found was that a two-injection was preferable to the four-injection model for two main reasons: it resulted in (i) a smaller lesion more localized to the mPFC, and (ii) less animal mortality. The two-injection model also has the advantage of a shorter surgical time, decreasing the amount of time that an animal is under anaesthesia, which may be part of the reason for the lower mortality rate with this group. The four-injection model was performed due to previous literature examining the effects of ibotenic acid, a neurotoxin used to lesion different parts of the brain in experimental animals, which has previously been used in a four-injection model to damage the mPFC (Birrell and Brown 2000; Risterucci et al. 2003; Shah and Treit 2003). However, the way ibotenic acid works is somewhat different than ET-1, in that the acid directly stimulates NDMA receptors, directly causing excitotoxic damage (Inglis and Semba 1997). ET-1 indirectly causes excitotoxic damage via vasoconstriction at the injection site, resulting in eventual ischemic conditions around the site of injection and throughout tissue fed by the constricted blood vessels. That may explain why the damage incurred through its injection is more extensive and diffuse than an injection of a similar volume of ibotenic acid.

Many areas of the brain have long been associated with the regulation of certain emotional states. The prefrontal cortex is thought to regulate many higher-order cognitive functions, as well as to regulate behaviours associated with changes in emotions (Uylings et al. 2003; Salzman and Fusi 2010). For example, damage to the

prefrontal cortex has resulted in changes in aggressive behaviours in rats as well as humans (Kolb and Nonneman 1974; de Bruin et al. 1983; Giancola 1995). As demonstrated in the results of this chapter, there appear to be changes in how animals with lesions to the mPFC respond to anxiogenic situations, in that animals with mPFC lesions were more likely to spend time in the closed arms of the maze as well as exhibit freezing behaviours. Other differences in behaviour that were exhibited by the mPFC lesioned animals included a lower number of rearing actions as well as less distance moved throughout the EPM trials.

The literature regarding prefrontal cortical manipulations on anxiety responses is mixed. One study demonstrated that animals with depleted levels of dopamine in the prefrontal cortex displayed anxiogenic-like behaviours, spending more time in the closed arms of the elevated plus maze (Espejo 1997). As in the current study, these animals also displayed decreased locomotor activity. However, further lesion experiments have not corroborated that evidence, and have given evidence counter to what was found in our study (Shah and Treit 2003; Blanco et al. 2009). These lesions, however, did not recapitulate the transient locomotor decreases that we observed in our model. Although many experiments demonstrate an anxiolytic response to lesions of the prefrontal cortex, experiments performed by others have demonstrated a lack of anxiolytic behaviour post-lesion of both the anterior cingulate and infralimbic cortices (Lacroix et al. 1998; Bissiere et al. 2006). Further research with our model may be required to better delineate the role of ischemic lesions to the mPFC and its effects in anxiety behaviours.

Although not specifically tested in this model, the behavioural changes seen in the elevated plus maze could be the result of damage to the prefrontal cortex, and areas outside of this region, affecting motor and/or sensory function. This could explain the transient reductions in movement in the stroke group between PSD8 and pre-surgery as well as PSD29, as well as the propensity for the increased amounts of time spent immobile. However, animals with chemically-induced lesions to the prefrontal cortex have exhibited increased levels of spontaneous movement or have produced no changes in movement overall (Carter and Pycock 1980; Swerdlow and Koob 1987). As well, previously cited research has demonstrated that rats with ibotenic lesions in the mPFC do not have changes in locomotor activity (Shah and Treit 2003). Further, post-hoc analyses of the data presented in this chapter via linear regression were performed examining the relative size of the lesion versus the amount of distance moved in the maze on PSD8, hypothesizing that a larger infarct may result in decreased locomotor activity. However, no correlation between the two values was found ($F_{(1,7)} = 0.4318$, $p > 0.05$, $R^2 = 0.058$). However, this analysis did not take into consideration the approximate locations of the damage, only the relative size of the area affected. Sensory deficits could also change behaviours within the elevated plus maze, but unfortunately sensory function was not assessed in these animals. Future experiments with this model of stroke induction should take motor and sensory function into account and a further battery of these tests should be employed post-surgery.

Temporal object recognition tests are used as a measure of temporal processing in animals, as a means of determining whether animals can sequentially list the order of certain items presented. This effect has been successfully demonstrated in human

subjects as well as many other vertebrate species (Shimp 1976; Kesner et al. 1984; Butters et al. 1994). For temporal order recognition, the prefrontal cortex has been demonstrated to be a necessary component of recalling the proper sequence of particular events (Chiba et al. 1997). In our task, the animals in both the sham and the stroke group failed to demonstrate measureable temporal order memory as seen in other experiments (Hannesson et al. 2004; Barker et al. 2007), indicating a potential flaw in the experimental process. This unexpected result demonstrated that particular aspects of the experimental process should be modified in order to demonstrate temporal order memory as found in other experiments. One of the possible reasons as to why the temporal order memory effect was not observed could be that the objects were not dissimilar enough for the animals to be able to differentiate. Although the Lego objects differed in overall size, shape, and colour pattern, each of the objects are similar in that they have identical textures (i.e. all objects were uniformly plastic). Previous experiments performed in other laboratories have used similarly constructed objects from Duplo blocks, and were able to demonstrate temporal order memory in control animals (Barker and Warburton 2011). However, with no diagrams or pictures from these tests on which to base our comparisons, it is unknown whether the constructed objects in our experiments differed significantly from the objects used in other experiments. As a result, subsequent experiments and experimental procedures were altered, and the results of these methodological changes are discussed in follow up studies that are presented in Chapter 3.

In conclusion, the experiments described in this chapter provide data that ET-1 lesions can be surgically induced in areas of the PFC with relative accuracy. Further, the

data presented in this chapter provides evidence that these induced lesions can affect cognitive function, although the reasons as to why certain behaviours were exhibited are unknown. Further experiments in chapters 3 and 4 explore the nature of other higher-order cognitive functions that these lesions may affect.

CHAPTER 3

HISTOLOGICAL AND BEHAVIOURAL EFFECTS OF ISCHEMIC LESIONS LOCALIZED TO THE MEDIAL PREFRONTAL CORTEX OF THE RAT

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SUMMARY

Ischemic stroke is one the leading causes of neurological disability worldwide, and it has been estimated that about one quarter of stroke survivors experience some measurable long-term impairments of cognition. Many higher order cognitive deficits occur because of damage to the prefrontal cortex (PFC), which is one of the main areas of the brain responsible for executive functioning in mammals. Currently, there are few animal models that attempt to replicate the effects of stroke on executive function. In this study we used bilateral micro-injections (1 µl) of the vasoconstricting peptide endothelin-1 (ET-1) into the medial PFC in male Sprague Dawley rats (or vehicle control, n = 17-18 per group) in order to model ischemic lesions in the medial PFC. The effects of these lesions on executive function were assessed using tests of set-shifting and temporal order recognition. ET-1 injections in the medial PFC (mPFC) resulted in replicable and specific lesions within the PFC with an average infarct volume of 16.63 ± 2.71mm^3. The ischemic lesions resulted in specific contextual set-shifting deficits within the maze, including an increased number of trials to criterion and a significant difference in learning curves. However, no deficits in temporal order memory processing were noted between sham and stroke animals. It was concluded that ischemic lesions localized to the mPFC result in selective but not generalized deficits in executive function in rats.

3.1. INTRODUCTION

Stroke is one of the leading causes of death and disability, with an estimated 795,000 new or recurring cases each year in the United States alone (Go et al. 2014). Although a majority of patients survive the initial cerebrovascular insult, many affected individuals have ongoing deficits in function that may persist for months or years post-stroke (Kelly-Hayes et al. 2003). The most common groups of deficits induced by stroke damage can be broadly classified into two general categories: motor deficits and cognitive deficits (Lawrence et al. 2001; Nys, van Zandvoort, de Kort, et al. 2005; Langhorne et al. 2009). Although new methods have been, and continue to be developed to treat post-stroke motor deficits, there are few demonstrably effective strategies to treat the many forms of cognitive dysfunction that may occur post-stroke (T. Hoffmann et al. 2010; Loetscher and Lincoln 2013). Further, patients suffering from cognitive deficits are often less receptive to therapies used to treat motor dysfunction, exacerbating the effects of stroke and hindering recovery (Winstein 1999; Jehkonen et al. 2006). Many types of post-stroke cognitive deficits have been described, including encoding-based deficits (Madureira et al. 2001), aphasia (Pedersen et al. 1995), and executive dysfunction (Zinn et al. 2007a). Deficits in executive function, which are considered higher order cognitive functions controlled by the prefrontal cortex, include working memory, goal-based decision making, and the learning and proper application of rules (Miller and Wallis 2009; Diamond 2013). In patients who have survived an initial stroke, it has been estimated that approximately one half have some measureable long-term deficit in executive function (Zinn et al. 2007b).

Although the mechanisms by which interrupted blood flow causes damage to the brain are relatively well understood, the potential deficits in behaviour resulting from this damage, as well as effective treatment strategies, are poorly understood and elusive. Furthering the current understanding in this regard relies heavily on the use of appropriate animal models (Minnerup et al. 2012; Hope et al. 2013). One of the most commonly used stroke models, the rat middle cerebral artery occlusion (MCAo) model, produces a replicable and reliable lesion both in the cortex and striatum, and results in both motor and some cognitive deficits (Belayev et al. 1996; Bouët et al. 2007). Unfortunately due to the size and location of the lesion, which is typically centred within the occipital lobe, the study of complex cognitive deficits in rats is confounded by overlapping sensory and motor deficits that probably contribute to the lack of documented reports on executive function deficits using MCAo (Carmichael 2005; Jiwa et al. 2010). Other commonly used stroke models, such as 2- and 4-vessel occlusion and hypoxia-ischemia, are similarly compromised in that the ischemic damage affects certain neuronal populations more than others, notably the CA1 region of the hippocampus, and photothrombotic models are largely restricted to surface vasculature (Kirino 1982; Traystman 2003; Schmidt et al. 2012). Recently, our laboratory and others have described how small well-localized ischemic lesions produced by surgical microinjection of endothelin-1 (ET-1) have been used successfully to produce discreet functional deficits in rats (Livingston-Thomas et al. 2013; Livingston-Thomas et al. 2014). Endothelin-1 is a potent vasoconstrictor capable of temporarily occluding blood vessels via an action on endothelin receptors (Sumner et al. 1992). This feature of temporary occlusion allows ET-1 injections to mimic the effects of a temporary

ischemic stroke. The compound can be injected directly onto blood vessels in the brain in order to mimic already established surgical protocols, or it can be injected within the brain parenchyma itself, resulting in small vessel occlusion and a consequent ischemic lesion directly at the site of application or injection (Fuxe et al. 1997; Windle et al. 2006). The ET-1 model is used throughout the studies described in this thesis.

Non-ischemic lesions occurring within the prefrontal cortex of rats can cause a variety of different behavioural effects, including temporal order memory dysfunction, working memory dysfunction, an attenuated fear response, alterations in social interaction, and deficits in attentional processing (Granon et al. 1994; Birrell and Brown 2000; Shah and Treit 2003; Hannesson et al. 2004). In humans, ischemic lesions in the dorso-lateral PFC, which is functionally equivalent to the medial PFC region in rats, cause a variety of functional deficits including an increased incidence in depressive symptoms and attentional set-shifting difficulties (Stuss et al. 2000; Thomas et al. 2003).

The objectives of this study were two-fold: first, to localize a stroke-induced lesion to the medial prefrontal cortex in rats via the injection of the vasoconstrictor ET-1, and second, to evaluate any resulting deficits in executive function using a temporal order memory task and a set-shifting task.

3.2 METHODS

3.2.1 Experimental Animals

All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved in advance by the University of Prince Edward Island Animal Care Committee, protocol number 12-035. Adult male Sprague-Dawley rats (n = 37, 250-275g on arrival) were purchased from Charles River Laboratories (Montreal, Canada) and singly housed on a reverse 12 h light/dark cycle (lights on at 18:00 and off at 06:00) with food (Purina rat chow) and water available *ad libitum* until training/testing (see below). Upon arrival, animals were acclimated to the facility for one week and were handled by the experimenter for 5 minutes each day for three days subsequent to the acclimation period. Subsequent to daily handling all animals were given 10 sucrose pellets (Bio-Serv, Frenchtown, USA) in their home cage in order to acclimate the animals to the reward. All behavioural training and testing occurred during the dark phase of the light cycle. All animals were weighed once daily.

3.2.2 Surgical Procedures

The surgical protocols for producing an ET-1 stroke lesion were as reported previously, with the exception of injection coordinates and volumes (Livingston-Thomas et al. 2013; Livingston-Thomas et al. 2014). Rats (ET-1 n = 19, sham n = 18) were anaesthetised by being placed into an induction box prefilled with 3.5% isoflurane (PPC,

Richmond Hill, Canada) in oxygen for 8 minutes, and anaesthesia was maintained throughout the surgery with a 2-3% isoflurane mixture. Animals were mounted on a stereotaxic frame (Kopf Instruments, Tujunga, USA), and the fur on the top of skull was shaved. Once shaved, topical Xylocaine (AstraZeneca, Mississauga, Canada) was applied to the shaved area and left for 5 minutes before a subsequent 2 cm midline incision was made down the top of the cranium. This incision was held open by 4 clamps. Two small burr holes were then drilled into the cranium above the coordinates required for drug injection. A 26 gauge 10 µl syringe (Hamilton, Reno, USA) was lowered into each of the injection sites (anterior/posterior +3.0, medial/lateral ±0.7, dorsal/ventral -4.5, all coordinates relative to bregma) and was left for 1 minute prior to ET-1 injection. After one minute, 1 µl of ET-1 (400 pmol) dissolved in artificial cerebral spinal fluid (aCSF) was injected into the cortex at a rate of 0.5 µl/minute. Once the ET-1injection was complete, the needle was left undisturbed for 4 minutes to allow for drug dispersal at the injection site, and then the needle was slowly retracted from the brain. After both ET-1 injections, the incision site was sutured and Xylocaine was reapplied. At the conclusion of the surgery each animal was given a 2.0 mg/kg subcutaneous injection of butorphanol tartrate (Wyeth, Guelph, Ontario) for post-operative analgesia. During surgical procedures, one animal could not be properly anesthetised and one animal died, therefore final group numbers for sham and ET-1 injections were n = 18 and n = 17, respectively. Sham treated animals received the same surgical treatment as stroke animals but received an equal volume injection of aCSF alone.

3.2.3 Set-Shifting Task

All animals were habituated and trained in the set-shift maze over a two week period prior to surgery (Section 3.2.2). The maze and testing protocol were modified from Stefani et al. (2003). The apparatus was a plus-maze constructed of Plexiglas walls (20 cm high) with metal arms (40 x 10.5 cm length and width). Each arm was lined with a Plexiglas insert which varied along two different stimulus dimensions: brightness or texture. The inserts were painted either black or white, with one arm of each colour painted with the inclusion of bird gravel (Hagen, Montreal, Canada) to create a rough texture. Each of the inserts spanned the full length of the arm, and the centre rectangle of the maze consisted of an 11.5 × 10.5 cm Plexiglas insert, painted grey (see Figure 3.1). The day prior to any training or testing, the animal was placed on a restricted feeding schedule, which consisted of 4 hours of *ad libitum* feeding followed by 20 hours of food deprivation. Once 20 hours had passed, the training or testing commenced and the animal was placed back on *ad libitum* feeding once the training or testing was complete. Of the animals in the experiment, five were excluded from the set-shifting task due to failing out of the training process.

3.2.3.1 SST training protocol

The training regimen prior to surgery was divided into two separate sections and was conducted over two weeks. The first week of training consisted of placing the animal in the maze with sucrose pellets in all of the arms and in each of the food cups

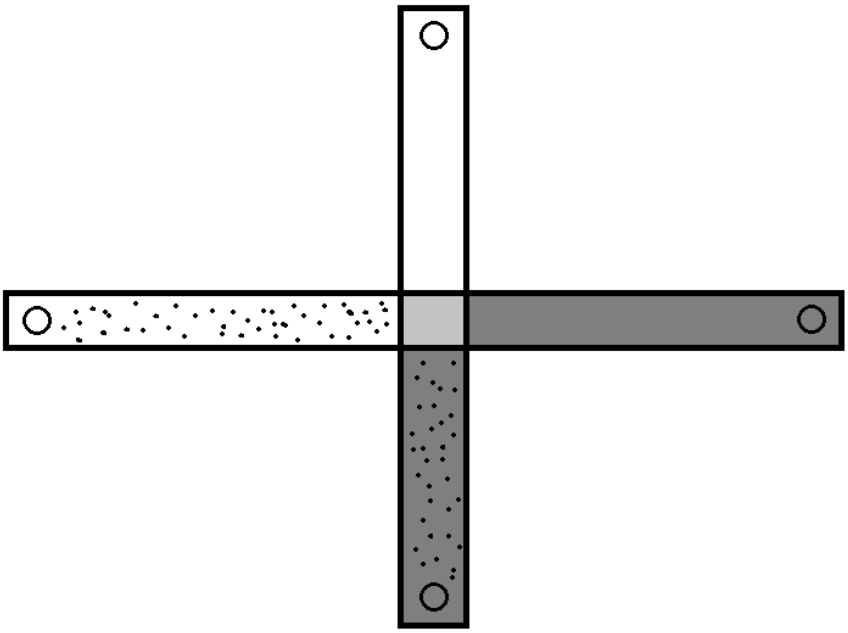


Figure 3.1. **A diagram of the attentional set-shifting maze.** During testing phases the animal is placed pseudo-randomly into one of the four arms, and the opposite arm is blocked creating a T-maze. The animal then has the choice to turn right or left to receive a food reward, depending on which stimulus (light, dark, rough, or smooth) is being rewarded.

located at the ends of the arms. The animals remained in the maze for 5 minutes or until each of the food pellets was consumed. Over the course of the first week the number of food pellets in the maze was reduced until the last day when there was only 1 pellet in each of the food cups.

The second week of training consisted of placing the animal pseudo-randomly into one of the four arms of the maze, and blocking access to the arm directly opposite the start arm, thereby creating a T-maze. The food cups of the right and left maze arms were each baited with 1 sucrose pellet, and the animal was allowed to choose 1 arm. A "choice" was defined as having all four limbs in contact with the Plexiglas insert within a choice arm. This procedure was performed 8 times in one training day, with the animal starting from each arm twice in one day. Between trials, the rat was placed into an inter-trial interval box (a standard animal holding cage with the exterior lined with black Bristol board) for approximately 15 seconds. Two days after completion of SST training the animals underwent the surgery as described in Section 3.2.2.

3.2.3.2 SST testing protocol

On post-surgery day 8 (PSD-8), the animals began SST testing by being trained to associate one feature of the Plexiglas inserts (brightness or texture) with the receipt of a food reward. Each rat was placed into one of the four arms with the opposite arm blocked, and allowed to choose right or left. Rats were randomly assigned to a brightness (light or dark) or texture (smooth or rough) group. Criterion was defined as choosing the correct arm, defined as the pellet-baited arm, on 8 successive trials up to a

maximum of 80 trials in one session. If 80 trials were completed without having reached criterion, they were removed and retrained to the same feature for an additional 80 trials, or until criterion was reached, the following day. The animal was pseudo-randomly placed twice into each of the arms over an 8 trial block, and the maze apparatus was rotated once every four trials. Four animals were unable to reach criterion in the task and were removed from this portion of the study.

For rats that achieved criterion, the stimulus associated with reward was reversed on the following day (i.e. rats that had learned to associate brightness with a food reward were now re-trained to associate a texture with the receipt of a food reward, and vice versa). Animals were tested for a minimum of 80 trials with success criterion, as defined previously (see above). Again, the animal was pseudo-randomly placed twice into each of the arms over an 8 trial block, and the maze apparatus was rotated once every four trials.

3.2.4 Temporal Object Recognition Task

Temporal object recognition (TOR) measures an animal's ability to distinguish between objects seen more recently versus objects seen less recently and has previously been reported to be dependent on an intact medial PFC (Barker et al. 2007). The maze apparatus consisted of a round, empty, fibreglass container (148.6 cm in diameter; walls 63.5 cm in height). To provide colour contrast for video recording the floor of the maze was covered in brown adhesive shelf liner.

On PSD5 and PSD6 each animal was acclimated separately to the maze for 10 minutes. Testing began on PSD 7 and consisted of placing the animal into the maze with a pair of identical objects positioned equidistant from the centre of the maze and the maze walls. The rat was allowed to explore for 4 minutes before being returned to its home cage. After a delay of 60 minutes, the animal was placed back in the maze with a new pair of identical objects for a further 4 minutes. After an additional delay of 180 minutes, the animal was returned to the maze with one object from each of the two previous trials for 3 minutes. Time spent exploring the objects was recorded via a digital camcorder and stopwatch, and active exploration of an object was defined as the animal facing the object and having its head within at least two centimetres of the object's surface. TOR testing took place on PSD 7, PSD 14, PSD 21, and PSD 28. Two sets of objects were used during this experiment, with one set of objects (a metal beam and a plastic box of nails) being used on PSD 7 and PSD 21, and the other set of objects (a flower pot and a rubber wheel) being used on PSD 14 and PSD 28.

3.2.5 Histology and Infarct Quantification

On PSD 28, rats were anaesthetized with isoflurane and euthanized by decapitation. Brains were removed and fixed in 10% formalin. Brain tissue was sectioned in 100μm segments by vibratome (Ted Pella, Redding, USA) and mounted on Surgipath X-tra glass slides (Leica Biosystems, Richmond, USA). Sections were subsequently stained with cresyl violet (0.1 % w/v). Images were prepared using a computer scanner (HP Scanjet 7650) at 2400 DPI resolution, and infarct quantification

was performed by tracing the area of tissue which displayed a lack of, or abnormal, cresyl violet staining or altered cytological architecture. Tracing was performed using ImageJ software, version 1.45s (ImageJ, National Institutes of Health, USA). Once each area had been traced, the volume of infarct damage was quantified by summing each individually calculated area and multiplying the area by the distance between each section (100 µm).

3.2.6 Statistical Analyses

Statistical analyses were performed using Graph Pad Prism, Version 5.00 (Graph Pad, La Jolla, USA). Weight differences between sham and stroke pre- and post-stroke were calculated using a repeated measures 2 way ANOVA with Bonferroni post-hoc tests comparing each group on each day.

In the first phase of the set-shifting task the number of trials to criterion was analysed using a one tailed Student's t-test, and the slopes of the learning curves (average number of correct choices by trial) were compared between all groups using a two-way ANOVA (sham/stroke × brightness/texture). For the second phase of the set shifting task the number of trials to criterion for each group were compared using a one tailed Student's t-test and the types of errors made by each of the trial groups were compared using separate two-way ANOVAs examining the types of errors versus the discrimination factor (brightness versus texture) for both the sham and stroke groups. In addition, the slopes of the learning curves (mean correct choices by block) were compared between groups using a two-way ANOVA.

The amount of time spent exploring each object in the temporal order recognition task was analysed using a two-way repeated measures ANOVA with post-hoc comparisons using t-tests with Bonferroni correction where appropriate.

In all analyses, results were considered statistically significant at $p \leq 0.05$.

3.1 RESULTS

3.3.1 Histology and Infarct Size

Bilateral ET-1 injections into the prefrontal cortex (Figures 3.2A and B) produced an infarct within the tissue, with the largest area of damage localized within the prelimbic and infralimbic cortices (approximately +3.0 anterior to bregma) (Figure 3.2C). The average volume of the infarct was found to be $16.63 \pm 2.71 mm^3$ (mean ± SEM). Data from two rats were not included in any analyses because one animal died during surgery and the other had an ischemic lesion that was significantly larger than the average; affecting regions well outside both the prelimbic and infralimbic cortices. Body weights between the two groups were compared on days PSD 0 through PSD 7 though a repeated measures two-way ANOVA, revealing no significant difference between the groups (Figure 3.3).

3.3.2 Behavioural testing

3.3.2.1 Set-Shifting Task

During the first phase of testing the number of trials needed to reach criterion (8 correct trials in a row) was recorded, and it was determined that there was no significant difference in the number of trials required by either the sham or stroke groups to correctly learn the association ($t_{(30)} = 0.271$; $p > 0.05$) (Figure 3.4A). As well, the slopes of the learning curves (mean correct choices by trial) from the first phase of the test were analysed through a two way ANOVA that compared both features (brightness and texture) as well as treatment groups (sham and stroke). There were no significant main effects for either the feature (brightness versus texture) ($F_{1, 26} = 3.01$; $p > 0.05$) or the surgical treatment ($F_{1, 26} = 1.34$; $p > 0.05$) and no significant interaction was found ($F_{1, 26} = 0.07$; $p > 0.05$), indicating that initial rates of learning the task did not differ between any of the groups.

Figures 3.2A-C. Overview of the surgical procedure and the resulting ischemic insult. (A) The approximate location of the two injection sites, denoted by the X. Picture adapted from the Rat Brain Atlas, 6th edition (Paxinos and Watson 2007). (B) A representative example of the ischemic insult found within the prefrontal cortex, stained with cresyl violet. (C) A line graph representing the relative area of damage at different stereotaxic levels (n = 17)

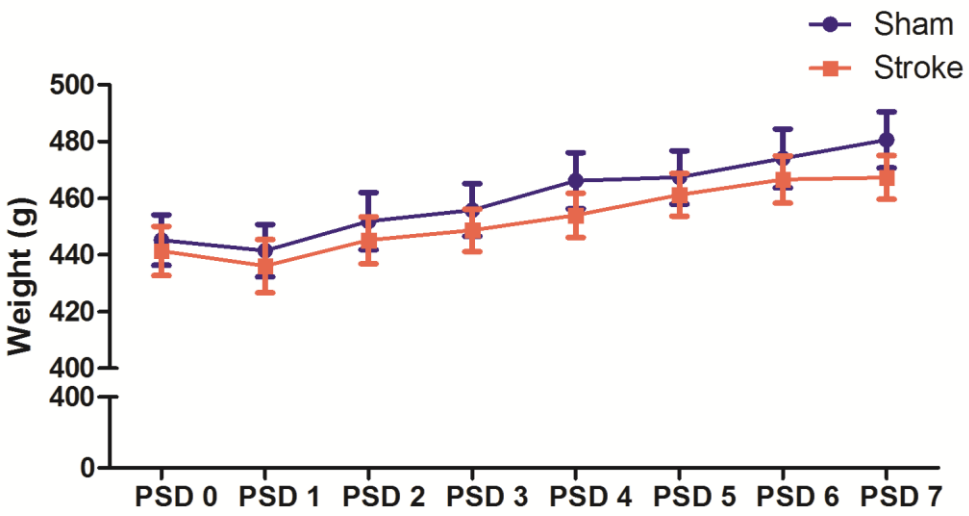


Figure 3.3 Animal weights pre- and post-surgery. A two-way repeated measures ANOVA found no significant differences between the sham (n = 18) and the stroke (n = 17) groups ($F_{1,33} = 0.395$ $p > 0.05$), nor an interactive effect found between the day weighed and the surgical group ($F_{7,33} = 1.59$ $p > 0.05$).

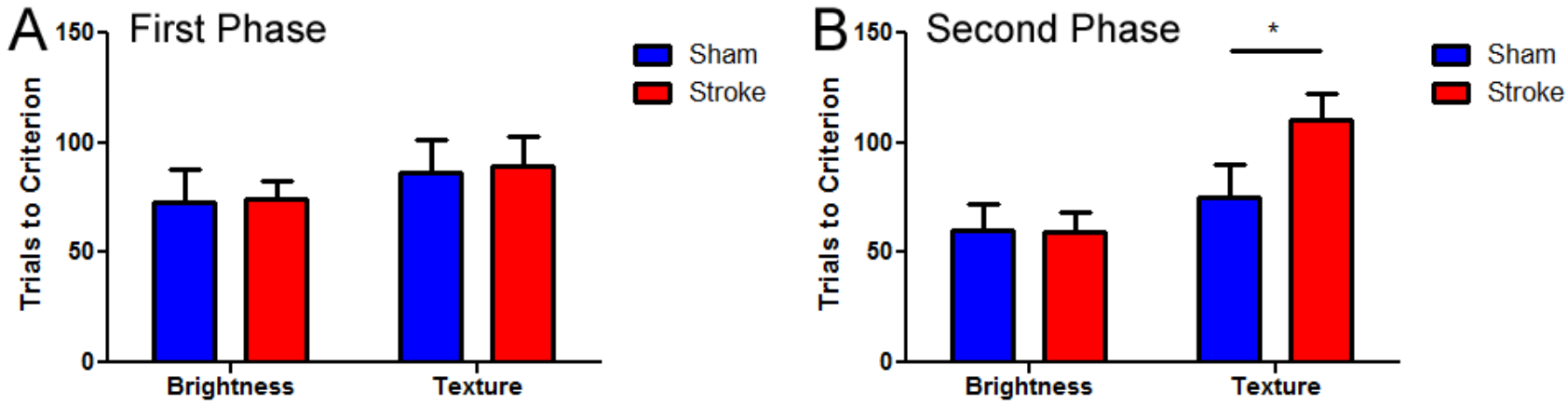
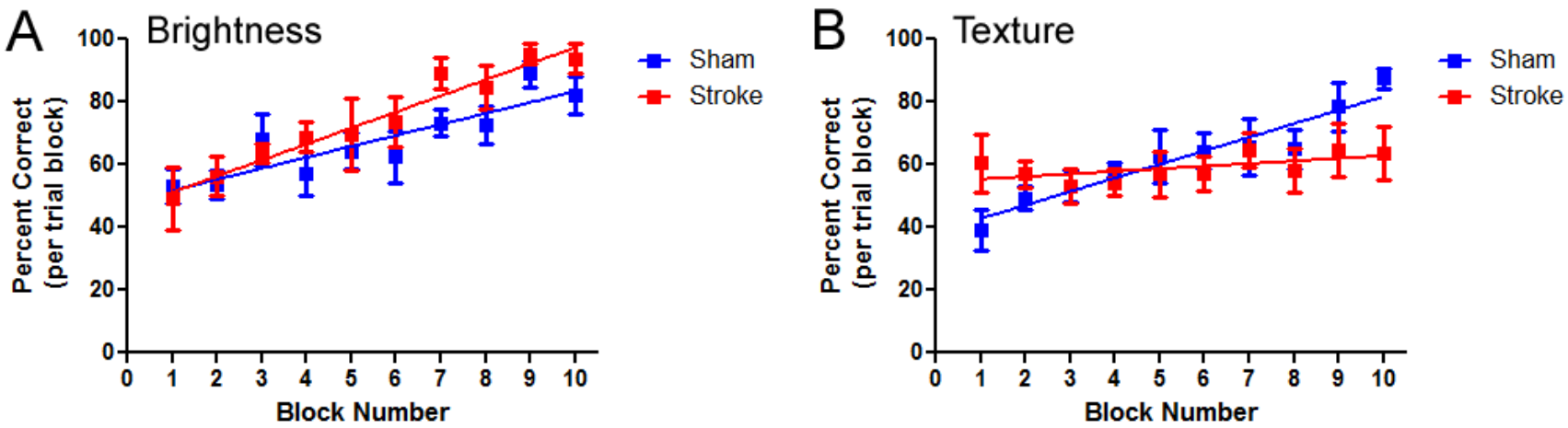


Figure 3.4A and B. An overview of trials to criterion to learn during the SST. (A) The number of trials needed to reach criterion during the first phase of the test, which is the initial association between a maze feature and receipt of a food reward. (B) The number of trials needed to reach criterion during the second phase of the test, which involves set-shifting from a previously relevant stimulous to a previously irrelevant stimulus. Though there was no difference between the number of trials needed for animals to set-shift to a brightness stimlus, there was a significant difference ($p < 0.05$) between sham ($n = 15$) and stroke ($n = 15$) animals in the number of trails required to set-shift to a texture discrimination.

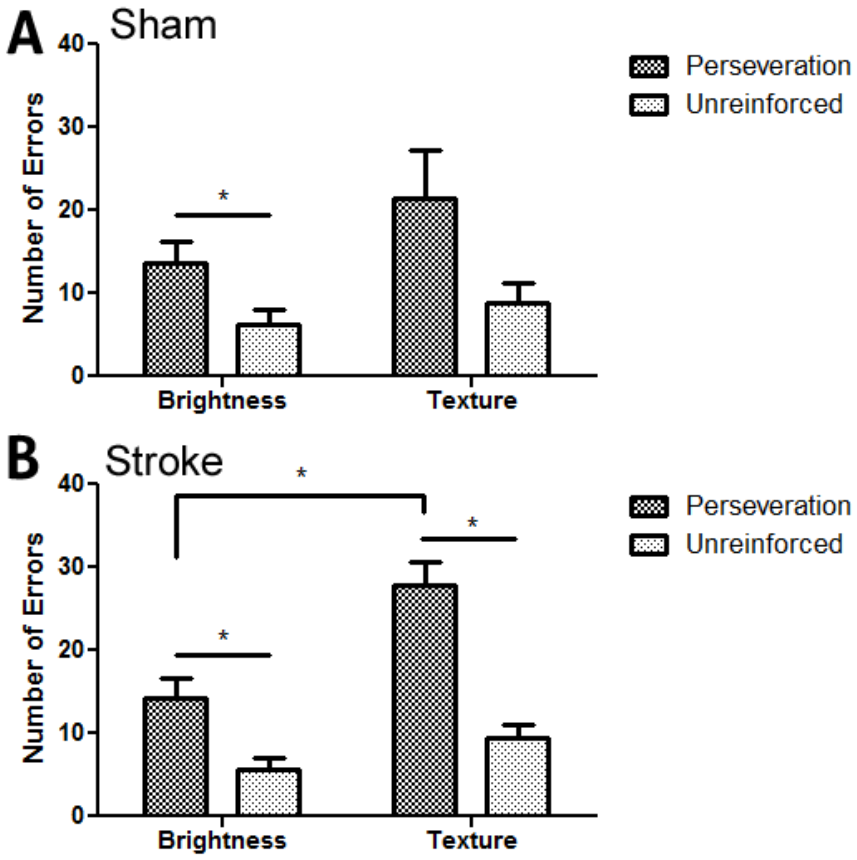
During the second phase of testing, stroke animals that had initially learned texture and then shifted to brightness learned the new association as quickly as sham-treated rats ($t_{(13)} = 0.02$; $p > 0.05$) (Figure 3.4B). However, animals lesioned using ET-1 that had originally learned the brightness feature were not able to learn the texture feature as quickly as their sham counterparts ($t_{(14)} = 1.82$; $p < 0.05$) (Figure 3.4B). The average learning curves of each of the groups of animals were also calculated. The results from the first 80 trials were amalgamated into 10 blocks of trials, with each block of trials representing the percent of correct choices the animal made over 8 trials (as depicted in Figures 3.5A and B). Once this was completed, the slope of the average learning curve for each group was calculated and compared between all groups using a two way ANOVA that revealed a significant main interaction effect between surgery group and maze feature ($F_{(3,28)}= 8.46$; $p < 0.01$). Subsequent post-hoc analyses revealed that there was no significant difference in the groups that shifted to the brightness feature (sham 3.51 ± 0.847, stroke 5.13 ± 0.704; $t_{(13)} = 1.48$; $p > 0.05$)(Figure 3.5A). However, there was a significant difference in the slope of the learning curves between sham and stroke animals that shifted from brightness to the texture feature (sham 4.50 ± 0.67, stroke 0.82 ± 1.18; $t_{(14)} = 2.61$; $p < 0.01$)(Figure. 3.5B).

To determine the nature of the association formed between cue and reward, data obtained during the second phase of the task were further analysed for both the number and type of error made by each animal. Incorrect choices made during this portion of the task were recorded as either perseveration or unreinforced errors, with perseveration



Figures 3.5A and B. Learning curves for the first 80 trials during phase 2 of the SST. Each block number represents an amalgamation of 8 trials into the percent of trials correct during that block. (A) Learning curves for the animals switching from texture to brightness. There was no significant difference in the learning curves of these animals (n = 7 for both sham and stroke groups). (B) Learning curves for the animals switching from brightness to texture. The average slope of the learning curve for the stroke animals was significantly less than the slope of the learning curve for the sham animals (n = 8 for both sham and stroke groups).

errors considered to be incorrect arm choices which would have been correct during the previous phase, and unreinforced errors considered to be incorrect arm choices that would have also been incorrect choices in the previous phase of the test. Within the sham group, there was a significant main effect between the number of perseveration errors versus unreinforced errors ($F_{(1,26)} = 7.49$; $p < 0.05$). Post-hoc analysis of these data revealed that sham-treated rats in the group switching to brightness commited significantly more perseveration errors than unreinforced errors (Figure 3.6A). There was no significant difference between the two types of errors within the group switching to texture although there was a tendency for more perseveration errors (Figure 3.6A). In contrast, in the rats treated with ET-1 there was a significant interactive effect when comparing the number of errors of each type in the brightness versus texture groups ($F_{(1,26)} = 4.92$; $p < 0.05$). Post-hoc analyses revealed that rats subjected to ischemic stroke committed significantly more perseveration errors whether they shifted to brightness ($t_{(13)} = 5.91$; $p < 0.001$) or to texture ($t_{(14)} = 6.44$; $p < 0.001$). As well, the total number of perseveration errors made between the texture-brightness and brightness-



Figures 3.6A and B. Overview of the types of errors made by each test group during the second phase of the SST test. (A) Types of errors in the sham group switching to the brightness feature or to the texture feature. (B) Types of errors in the stroke group switching to the brightness feature or to the texture feature. n = 7 for each brightness group (sham and stroke) and n = 8 for each texture group (sham and stroke) *$p < 0.05$.

texture groups was significantly different, with rats in the brightness-texture group committing more errors of this type ($t_{(13)} = 3.63$; $p < 0.01$) (Figure 3.6B).

3.2.2.2 Temporal Object Recognition Task

Data obtained over 4 weeks post-surgery in the TOR task are summarized in Tables 3.1-3.3. There were no significant differences in the amount of time the animals spent examining the first or second object in either the sham or stroke groups on any of the test days (PSD 7 $F_{(1, 33)} = 2.85$; PSD14 $F_{(1, 33)} = 1.32$; PSD21 $F_{(1, 33)} = 0.31$; PSD28 $F_{(1, 33)} = 4.21$; $p > 0.05$ for all testing days)(Table 3.1) indicating that overall both groups of animals were unable to distinguish between objects presented first or objects presented second, although there was a tendency in both groups to prefer the object presented first (Table 3.1).

Prior to testing, preliminary evaluation of the objects used indicated that normal rats had no innate preference for one object over the other (data not shown). In order to determine if ischemic stroke altered object preference, the data were separated into total time spent with a specific object, summarized in Tables 3.2A and 3.2B. Neither group of animals displayed a significant object preference on either PSD 7 or PSD 21 (PSD7 $F_{(1,33)} = 1.51$; PSD21 $F_{(1,33)} = 3.73$; $p > 0.05$ for both testing days)(see Table 3.2A). However, there was a significant main effect between object preference on PSD 14, due to rats in the stroke group (but not sham) spending more time with the flower pot than the wheel (PSD 14 $F_{(1,33)} = 10.3$; $p < 0.01$)(see Table 3.2B). On PSD 28, there was a

Table 3.1 Mean (+/- SEM) time (seconds) spent examining the objects presented in the temporal order test over three minutes. Neither test group spent significantly more time with the first object presented over the second object presented for any of the days tested. Sham n = 18, stroke n = 17.

	Sham		Stroke	
Testing Day	First Object	Second Object	First Object	Second Object
PSD 7	19.7s ± 2.45s	16.6s ± 1.66s	22.1s ± 2.00s	18.0s ± 2.51s
PSD 14	15.2s ± 1.57s	12.5s ± 1.55s	17.7s ± 3.40s	14.2s ±3.33s
PSD 21	20.3s ± 2.22s	19.0s ± 2.26s	25.9s ±3.14s	21.3s ±2.27s
PSD 28	12.4s ± 1.66s	9.17s ± 1.30s	17.2s ±3.55s	10.9s ±2.38s

Table 3.2A. Mean (+/- SEM) time (seconds) spent examining specific objects in the maze (a metal beam versus a plastic box of nails) on PSD 7 and PSD 21. There was no significant difference in the amount of time each group (sham and stroke) spent examining one object versus the other on either of the days that they were tested. Sham n = 18, stroke n = 17.

	Sham		Stroke	
Testing Day	Metal Beam	Plastic Box	Metal Beam	Plastic Box
PSD 7	21.5s ± 2.27s	17.9s ± 1.71s	19.5s ± 2.66s	17.6s ± 2.18s
PSD 21	23.0s ± 2.45s	18.1s ± 1.76s	25.9s ± 3.14s	21.3s ± 2.27s

Table 3.2B Mean (+/- SEM) time (seconds) spent examining specific objects in the maze (an empty overturned flower pot versus a rubber wheel) on PSD 14 and PSD 28. On both test days rats in the stroke group spent significantly more time exploring the flower pot than the wheel; an effect also seen in the sham group on PSD 28. *$p<0.05$. Sham n = 18, stroke n = 17.

	Sham		Stroke	
Testing Day	Flower Pot	Wheel	Flower Pot	Wheel
PSD 14	15.6 ± 1.52	11.9 ± 1.52	22.0 ± 4.11	9.98 ± 1.36 *
PSD 28	13.7 ± 1.59	7.85 ± 1.05*	21.4 ± 2.98	6.77 ± 1.15 *

significant interactive effect between the sham and stroke groups and the amount of time spent with each object (PSD 28 $F_{(1,33)} = 5.49$; $p < 0.05$). To compare time spent between flower pot and wheel, post-hoc paired t-tests were used comparing both differences in exploration between the sham group object preference and stroke group object preference. There was a significant difference between exploration times in both groups (Sham $t_{(18)} = 3.781$; $p < 0.01$); (Stroke $t_{(17)} = 4.331$; $p < 0.001$). When comparing flower pot and wheel exploration times between treatment groups via unpaired t-tests, there was a significant difference in the amount of time spent exploring the flower pot ($t_{(33)} = 2.261$; $p < 0.05$). However, there was no difference in the amount of time spent exploring the wheel ($t_{(33)} = 0.6910$; $p > 0.05$).

The same data were also analysed to determine the discrimination ratio between object types (flower pot versus rubber wheel, metal box versus nail box). This value was calculated by averaging the amount of time spent with each object by each animal, and dividing the subtraction of one from the other by the total amount of time spent exploring both objects. Each set of values was then analysed through a one-sample t-test with a theoretical mean of 0. Each value on PSD 14 and PSD 28 was significantly different from the theoretical mean, indicating that animals spent more time exploring one object (the flower pot) versus the other (rubber wheel) during the task(PSD 14 Sham [$t_{(16)} = 2.520$; $p < 0.05$]; PSD 14 Stroke [$t_{(16)} = 4.236$; $p < 0.001$]; PSD 28 Sham [$t_{(16)} = 4.279$; $p < 0.001$]; PSD 28 Stroke [$t_{(16)} = 5.747$; $p < 0.001$]). (see Table 3.3).

Table 3.3 Ratio scores comparing the amount of time spent with each object in the temporal object recognition maze. Sham n = 18, stroke n = 17.

Testing Day	Sham	Stroke
PSD 7	0.065	0.017
PSD 14	0.202*	0.280*
PSD 21	0.115	0.098
PSD 28	0.305*	0.469*

3.4. DISCUSSION

This study investigated the histological and behavioural effects of bilateral ET-1 injections to the medial PFC. The protocol resulted in consistent and well-localized ischemic lesions that appeared to have no effect on associative learning, relative to surgical sham controls, but did have a differential effect on the set-shifting ability of ET-1 treated rats. Lesioned animals switching from a texture feature to a brightness feature performed equally to their sham counterparts, but stroke animals switching from a brightness feature to a texture feature were neither able to shift strategies as quickly nor learn as quickly as sham animals. ET-1 injections in the medial PFC did not have a significant effect on an assessment of temporal order memory.

Histologically, the ET-1 injection protocol employed herein resulted in lesion volumes similar to a 2014 report by Cordova et al (2014) who used four ET-1 injections (as opposed to two) into the medial prefrontal cortex but a smaller volume of ET-1 per injection (0.8 µl versus 1.0 µl) although the concentration of ET-1 was not reported. Those authors did report that ET-1 induced lesions in the medial PFC significantly altered performance in one of the measures examined in a test of attentional set-shifting originally described by Birrell and Brown (2000). In that version of the test, animals are tested in a variety of cognitive tasks by having to burrow through digging media in flower pots that are assigned a particular smell. This method of testing includes an attentional set-shifting task, as well as a simple discrimination task, a compound discrimination task, an intra-dimensional shift task, and a reversal learning task. In their

experiment, stroke animals performed comparably to control animals, except when required to switch from an odour stimulus to a texture stimulus. In the current study we found a similar context-dependent effect on set-shifting. Rats with medial PFC localized lesions displayed no difference in how quickly the animals initially learned either the brightness or the texture features. When required to shift the relevant context feature, stroke animals switching from brightness to texture were significantly impaired whereas those switching from texture to brightness were not (Figures 3.3 and 3.4). This contrasts data reported by Stefani et al. (2003) who did not describe any differences in brightness/texture versus texture/brightness learning in a comparable task. However, these authors were not specifically studying ischemic stroke, but rather were selectively inhibiting excitatory neurotransmission in the medial PFC through the use of injections of glutamate receptor antagonists. The ischemic lesions produced in the current study would damage multiple transmitter systems as well as axons of passage and, consequently, might have differential effects on behaviour.

One possible reason for the discrepancy between brightness and texture set-shifting observed may be the nature of the stimulus itself. During the first portion of the task, the animals had no predisposition to either brightness or texture. But after having learned that one feature is associated with reward whereas the other is not, rats would be inclined to make their choice as quickly as possible in order to be rewarded. When an animal enters the middle of the maze, they see the brightness of each arm and can immediately make a choice based on that feature. This is different to how animals would distinguish texture, for in order to do that they must first enter the arm to feel the texture

before they are able to distinguish a right or wrong choice. Therefore, there is a difference as to the timing of these two stimuli. Consequently, if an animal is prone to making impulsive decisions, animals may be better able to follow brightness cues as they are more quickly available in contrast to texture cues. Interestingly, it has been observed that damage to the prefrontal cortex has been previously associated with increased impulsivity in both rats (Mobini et al. 2002; Rudebeck et al. 2006) and humans (Bechara 2000; Berlin et al. 2004). This interpretation may not be able to explain the reasons why Cordova et al. (2014) found no dimension-specific differences within their experiment, despite having a similar surgical protocol and lesion size. However, this may be due to discreet differences in the methodologies in our experiments; our experimental protocols require only a two-step process (initial learning and an extradimensional shift), wherein their intradimensional (ID)/ extradimensional (ED) experimental design required simple and compound discriminations as well as a reversal learning task, which required the animals to undergo multiple learning processes. To determine if impulsivity is in fact a contributing factor to the results obtained, future experiments could be refined to test either the impulsivity of rats following an ischemic lesion to the medial PFC, or to modify aspects of this task to use other sensory systems, including odor or auditory stimuli. Another potential explanation for the discrepancy between stroke animals learning differently between the brightness and texture features is the nature of the training regimen. It has previously been noted that deprivation of water affects how animals are able to learn optimal shift discrimination tasks, specifically in the context of brightness versus texture cue utilization (Cohen and Hachey 1977). Although the deprivation schedule within this task

was food-based rather than water-based, different types of deprivation may alter an animal's ability to switch to relevant reward cues in their environment.

One of the potential limitations of this study is that the test employed could be considered a test of simple set-shifting rather than attentional set-shifting, similar in nature to plus-maze experiments requiring a change from an egocentric response to a visual cue-based strategy (Floresco, Ghods-Sharifi, et al. 2006). In a typical ID/ED shifting task, such as the one developed by Birrell and Brown, the number of trials typically required to perform the ED shift is more than the ID (2000). However, it has been reported under different experimental conditions using this task that the number of trials required to switch between intra and extradimensional shifts can remain the same (Lapiz and Morilak 2006). For the texture/brightness ED shift in the SST, both groups of animals performed equally, not worse, during the initial learning task. This indicates that an attentional set may not have been formed. However, in the brightness/texture discrimination portion of the task, lesioned animals had considerable difficulty strategy-shifting compared to the sham animals, who performed equally well between the first and second phase of the test. The difficulty for stroke-lesioned rats to perform the brightness/texture shift is supported by the data in Figure 3.5. These data reveal that rats in all groups committed more perseverance errors than unreinforced errors, which can be interpreted as evidence of forming a 'set' during the first phase. However, in the rats that received ET-1 the number of perseverance errors within the brightness/texture stroke group was found to be significantly higher than the texture/brightness stroke group (Figure 3.5B). Although the issue of whether the results support an attentional shift or a strategy shift is hard to resolve in the test employed, as stroke animals do

appear to have some difficulties switching between a visual and a texture based reward cues in this paradigm. This suggests that the SST is an ideal task to test an animal's ability to set-shift between different reward cues following ischemic lesions.

The effects of a localized ischemic lesion to the medial PFC on temporal order memory remain unresolved. During the task, both groups of animals spent equal amounts of time with both objects (see Table 3.2A and B), as opposed to the expected effect of a preference based on how recently the object was presented, as described by others (Mitchell and Laiacona 1998; Barker et al. 2007). One reason for this lack of effect may have been the size of the test arena (148.6 cm in diameter), as this may reduce the total time spent exploring the objects. Otherwise, the conditions employed were comparable to those used by other authors (Mitchell and Laiacona 1998; Barker et al. 2007). Another reason why the animals may not be able to distinguish the first presented object from the second is due to the amount of delay time between initial presentation of the objects and the test phase, which in this study was three hours. Other studies examining object recognition have found exploration time differences between novel and familiar objects at 15 minutes, but not at 4 hours (Hotte et al. 2005). However, under similar conditions other studies suggest that temporal object recognition may be a relatively robust phenomenon, with differences in exploration time being reported 24 hours after initial presentation of the objects (Mitchell and Laiacona 1998). One particularly intriguing finding, however, was that despite prior pilot testing (that determined a lack of preference for the objects used; results not shown), during the test sessions there was a notable but not significant tendency for sham-treated rats to prefer the flower pot to the rubber wheel (see Tables 3.2A and B); a tendency that became

statistically-significant following ischemic stroke on PSD 14. Such a difference was not noted with the other objects used (metal beam and box) and an obvious explanation for the observation remains elusive.

Our results add to a growing body of literature that supports a complex role for the prefrontal cortex in executive function in rats. Different areas of the prefrontal cortex can control different functions and different areas of the brain connecting to an intact prefrontal cortex can also affect executive functions. The dorsomedial thalamus, a brain region with efferent connections to the prefrontal cortex, has been demonstrated to be involved with attentional processing and shifting attention to new rules (Hunt and Aggleton 1998). In a recent study, however, injections of ET-1 to the dorsomedial thalamus appeared not to affect attentional set-shifting, although the authors speculate that this could have been the result of insufficient ischemic damage to this region (Cordova et al. 2014). Interestingly, another factor which has an effect on attentional set-shifting function and possibly the function of the medial PFC, is the age of the animal. One study described the ability of young (4-5 month) vs old (27-28 month) Long-Evans rats in an attentional set-shifting task, with the younger rats outperforming the older rats, achieving criterion in fewer trials than older rats (Barense et al. 2002). As well, the aged rats showed no deficits in either reversal learning or spatial memory, suggesting that the deficit in function could be localized to the medial PFC. Although their study described no detectable histological differences between young and old animals, it was hypothesized that this difference could have been due to decreases in glutamate receptors within the PFC. Compounding this issue is the fact that the brain is

less resilient to ischemic insult as it ages, which could prove detrimental to recovery efforts for older individuals suffering from ischemic damage (Davis et al. 1995).

In summary our data indicate that ET-1 injections within the brain can be localized to specific subregions of the prefrontal cortex, resulting in targeted and reproducible ischemic lesions to the medial PFC. Such lesions appear to affect specific aspects of cognitive behaviour in rats but do not generalize to global deficits in executive function. Further experimentation on other aspects of cognitive behaviour, including impulsivity, effort-based decision making, and aspects of social competencies in rats, is needed to unravel the apparently complex role of the PFC in appetitively-motivated decision making, and to guide future research into cognitive rehabilitative strategies following stroke.

CHAPTER 4

EFFECTS OF PREFRONTAL CORTICAL LESIONS ON INHIBITORY CONTROL AND ULTRASONIC VOCALIZATIONS IN THE RAT

SUMMARY

Stroke is one of the most prominent causes of neurological disability in the world, and the number of cases occurring worldwide is expected to increase of the coming decades. As such, new mechanisms for combating this disease will be increasingly necessary. One of the issues with the treatment of stroke is that there are few methods by which we can help alleviate the cognitive deficits caused by this disease. As well, there are very few pre-clinical models of stroke with a specific focus on higher-order cognitive functions. The goal of the experiments performed in this chapter of the thesis was to examine the effects of ischemic lesions to the medial and orbital prefrontal cortices on inhibitory control, an important aspect of executive function, and ultrasonic vocalizations, which give an indication as to the affective state of the animal. Adult male Sprague-Dawley rats were tested in a delay discounting maze to examine their inhibitory behaviour, as well as to record and analyse ultrasonic vocalizations made during the test. It was found that animals with lesions to the orbital prefrontal cortex (PFC) displayed deficits in inhibitory control compared to their sham and medial PFC lesioned counterparts at multiple time points. In contrast, the medial PFC lesioned animals displayed differences in vocalization duration and frequency as compared to orbital PFC and sham controls. The results presented in this chapter warrant further research into the effects of localized prefrontal ischemic lesions and offer possibilities for improved pre-clinical modelling of post-stroke cognitive outcomes.

4.1 INTRODUCTION

Stroke is one of the most common causes of death and disability worldwide. In the United States alone, it is estimated that on an annual basis approximately 800,000 individuals will suffer a stroke, and the number of individuals having been afflicted by this disease is also set to rise as the population ages (Mozaffarian et al. 2014). This will result in large increases in direct and indirect health care costs, which are expected to rise from $71.6 billion dollars in 2012 to $184.1 billion dollars in 2030 (Ovbiagele et al. 2013). As such, new treatment strategies need to be developed to help ease this economic and social burden.

One of the more insidious results of an acute stroke is the detrimental effects on cognitive function. A number of studies have been performed examining the prevalence of cognitive dysfunction post-stroke. A study from Madureira et al. (2001) estimated that 55% of stroke survivors had deficits in at least one cognitive domain while undergoing the Mini Mental State Exam (MMSE). Other studies have shown similar findings, with Pohjasvaara et al. (2002) estimating that over 40% of stroke survivors exhibit executive function deficits, and Rasquin et al (2004) demonstrating that over half of stroke survivors show at least mild cognitive impairment (MCI) one year post-stroke. Not only does cognitive dysfunction post-stroke affect the patient's mental faculties, but these declines in cognition can also affect the patient's ability to successfully complete other rehabilitation programs, which in turn affects their overall ability to function in everyday life (Galski et al. 1993).

One of the higher-order cognitive functions that can be negatively affected through stroke or other brain lesions is inhibitory control, which is crucial to proper decision making as well as emotional control (Starkstein and Robinson 1997). Patients with deficits in proper inhibitory control often demonstrate difficulties in a wide variety of behaviours and tasks, including deficits in Go/No-Go executive function testing and behavioural disinhibition, and can demonstrate severe emotional incontinence (Aybek et al. 2005; Tang et al. 2009). At this time, there are few treatment options available for those patients who suffer loss of behavioural control.

Previous animal studies examining the effects of modelled prefrontal cortex (PFC) damage on inhibitory control report various findings as to the brain regions required for this behaviour. For example, recent evidence suggests that both the medial and orbital prefrontal cortices are required for impulsive choice control as measured in a delay discounting task (Yates et al. 2014). However, other findings suggest that inhibitory control can be traced to specific areas of the PFC, depending on how the behavioural tasks testing impulsivity are administered (Rudebeck et al. 2006; Churchwell et al. 2009; Mar et al. 2011). As such, we chose to explore the effects of ischemic prefrontal lesions in the medial PFC and orbital PFC on impulsivity, which may result in new methodologies for modelling the effects of stroke on complex cognitive dysfunction. The results of these experiments could facilitate further studies on cognitive or pharmacological rehabilitation post-stroke.

Another aspect of cognitive function and rat behaviour that was examined was the effects of ischemic lesions on ultrasonic vocalization behaviour. Generally, adult rats emit ultrasonic vocalizations under specific sets of conditions, and the general frequency

and duration of these vocalizations depends on the context and situation of the experiment. For example, rats can emit long and relatively low-frequency calls (22 kHz) in response to aversive stimuli, such as the presence of a predator or experimentally elicited pain (Blanchard et al. 1991; Calvino et al. 1996). Conversely, rats also emit relatively short, higher frequency calls (50 kHz) in response to relatively positive stimuli, such as during experimental-induced tickling, amphetamine injection and reward expectation (Burgdorf et al. 2001; Mällo et al. 2007; Brenes and Schwarting 2014). These 50 kHz calls are thought to indicate a positive appetitive affective state.

The behavioural task performed in our series of experiments testing inhibitory control provided an excellent opportunity to study elicited 50 kHz calls in anticipation of a food reward. The potential effects of ischemic lesions in areas of the PFC on vocalization behaviour have not been extensively studied, and this research may be able to give new insight into the affective state of rats with PFC lesions. As such, we performed ultrasonic recordings during the experimental procedures in an attempt to determine the effects of specific lesions in the PFC on ultrasonic vocalization behaviour.

The central purpose of this study was to investigate whether focal ischemic lesions within specific areas of the prefrontal cortex, specifically the medial and orbital prefrontal cortices (mPFC and oPFC, respectively), would affect inhibitory control behaviour as measured through a specific delay discounting paradigm. The hypothesis was that animals with lesions in the prefrontal cortex would choose the immediately available, lower reward option more frequently than control animals.

As a secondary experiment involving the delay discounting paradigm, the effects of prefrontal cortical lesions on ultrasonic vocalizations while performing the

behavioural experiment was also assessed, with the hypothesis that prefrontal ischemic lesions would affect aspects of duration, frequency, and number of calls made by lesioned animals, indicating changes in affective state.

4.2 METHODS

4.2.1 Experimental Animals

All procedures were conducted in accordance with the guidelines of the Canadian Council for Animal Care and were approved in advance by the University of Prince Edward Island Animal Care Committee, protocol number 12-035. Adult male Sprague-Dawley rats (n = 37, 250-275g on arrival) were purchased from Charles River Laboratories (Montreal, Canada) and singly housed on a reverse 12 h light/dark cycle (lights on at 18:00 and off at 06:00) with food (Purina rat chow) and water available *ad libitum* until training and testing. Upon arrival, animals were acclimated to the facility for 5 - 7 days and were handled by the experimenter for 5 minutes each day for three days during the acclimation period. Subsequent to daily handling, all animals were given 10 banana-flavoured sucrose pellets (Bio-Serv, Frenchtown, USA) in their home cage in order to acclimate the animals to the reward. All behavioural training and testing occurred during the dark phase of the light cycle. During the training and testing phases, animals were maintained at approximately 90% of their initial free feeding weight.

4.2.2 Surgical Procedures

The surgical protocols for producing an ET-1 stroke lesion were as reported previously (see Chapter 3, Materials and Methods), with the exception of injection coordinates and volumes. The rats were anaesthetised by being placed into an induction box prefilled with 3.5% isoflurane (PPC, Richmond Hill, Canada) in oxygen for 8 minutes, and anaesthesia was maintained throughout the surgery with a 2-3% isoflurane mixture. Animals were mounted on a stereotaxic frame (Kopf Instruments, Tujunga, USA), and their heads were shaved. Once shaved, topical Xylocaine (AstraZeneca, Mississauga, Canada) was applied to the shaved area and left for 5 minutes before a subsequent 2 cm midline incision was made down the top of the cranium. This incision was held open by 4 clamps. Two small burr holes were then drilled into the cranium above the coordinates for drug injection. A 26 gauge 10 µl syringe (Hamilton, Reno, USA) was lowered into each of the injection sites according to coordinates listed in Tables 4.1 and 4.2 for mPFC and oPFC, respectively, and was left for 1 minute prior to ET-1 injection (see Figure 4.2A and B for injection diagrams). After one minute, 1 µl of ET-1 (400 pmol) dissolved in artificial cerebral spinal fluid (aCSF) was injected into the cortex at a rate of 0.5 µl/minute. Once injected, the needle was left undisturbed for 4 minutes to allow for drug dispersal at the injection site, and then the needle was slowly retracted from the brain. After both ET-1 injections, the incision site was sutured and Xylocaine was reapplied. At the conclusion of the surgery each animal was given a 2.0 mg/kg subcutaneous injection of butorphanol (Wyeth, Guelph, Ontario) for post-

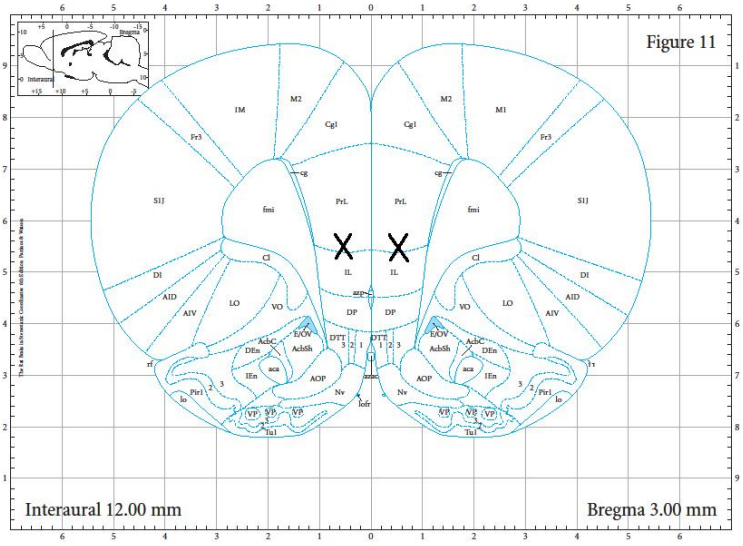
Table 4.1 Surgical Injection Coordinates Targeting the mPFC. Injection coordinates for endothelin-1 in the mPFC. A/P = anterior/posterior, M/L = medial/lateral, D/V = dorsal/ventral, with distances in mm relative to bregma. n = 10.

	A/P (mm)	M/L (mm)	D/V (mm)	Volume (µl)
Injection 1	+3.0	-0.7	-4.5	1
Injection 2	+3.0	+0.7	-4.5	1

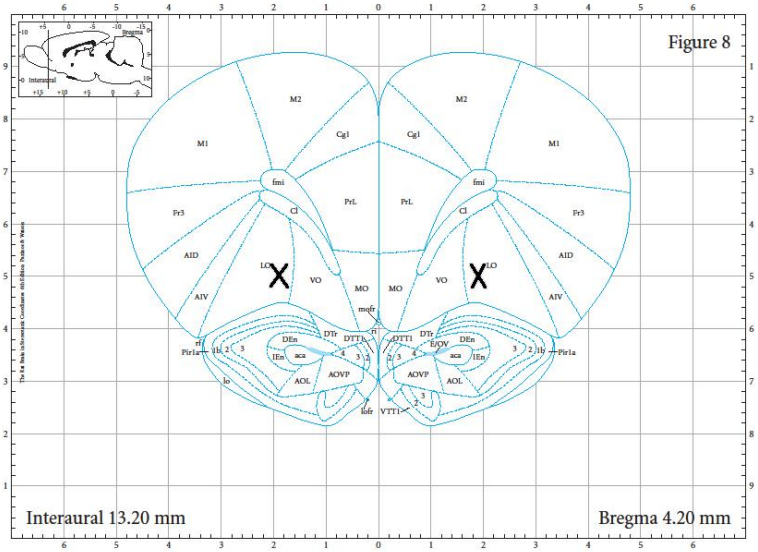
Table 4.2 Surgical Injection Coordinates Targeting the oPFC. Injection coordinates for endothelin-1 in the oPFC. A/P = anterior/posterior, M/L = medial/lateral, D/V = dorsal/ventral, with distances in mm relative to bregma. n = 11.

	A/P (mm)	M/L (mm)	D/V (mm)	Volume (µl)
Injection 1	+4.0	-2.0	-5.0	1
Injection 2	+4.0	+2.0	-5.0	1

A



B



Figures 4.1A and B. Cartoon figures adapted from Paxinos and Watson (2007)**.** Each marked "X" represents a separate injection point. (A) represents the injection points for the mPFC ET-1 injections (n = 10), and (B) represents the oPFC ET-1 injections (n = 11).

operative analgesia. Sham treated animals received the same surgical treatment as stroke animals but received an equal volume injection of aCSF.

4.2.3 Behavioural Testing

4.2.3.1 Delay Discounting

The experimental procedures and maze apparatus were adapted from experiments published by Rudebeck et al. (2006). The maze consisted of a re-adapted and modified Pathfinder Maze System radial-arm maze (Figure 4.2) (Lafayette Instruments, Lafayette, USA). Briefly, the radial arm apparatus was modified to the shape of a T-maze (one stem arm, two choice arms), with each of the metal arms being 71 cm in length, 10.5 cm in width, and with Plexiglas® walls 20 cm in height. Black Bristol board covered the exterior portions of the choice arm walls. Each choice arm had a small food cup crafted within the floor of the arm.

Training of the animals consisted of four days of maze acclimation, wherein the animal would be placed at the stem of the T-maze with all maze gates open. Dustless banana sucrose pellets (45 mg; Bio-Serv, Frenchtown, USA) were placed within the arms and food cups, with 10 pellets placed in the left arm, and 1 pellet in the right arm. The animals were left to explore the entire maze until all pellets were consumed or until 10 minutes had passed. Over the course of four days, the number of pellets strewn within the arms was reduced until there were only pellets within the

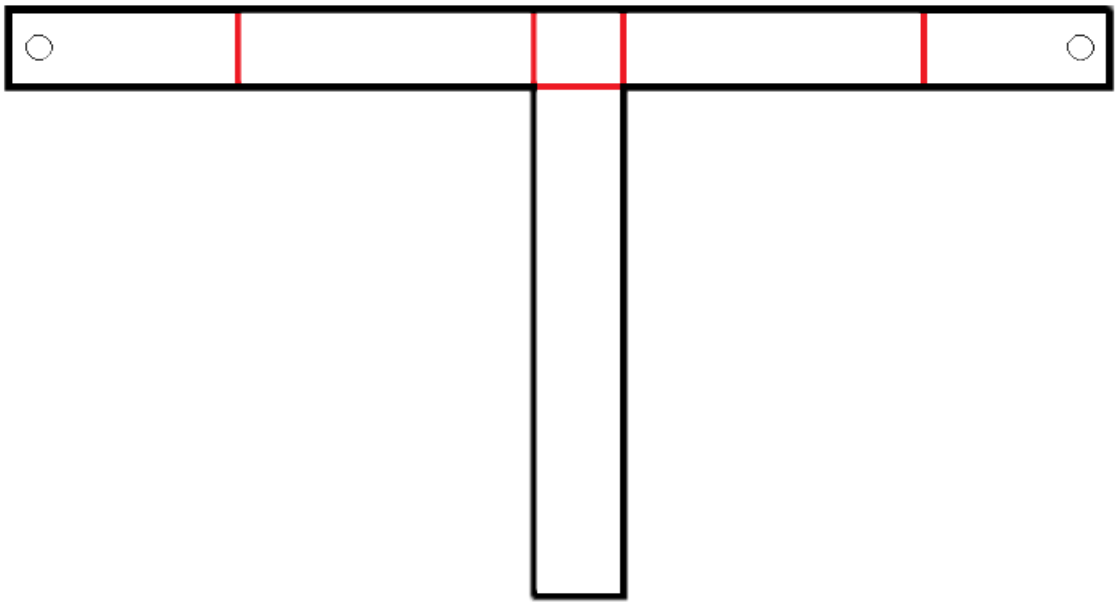


Figure 4.2 An overhead representation of the delay discounting maze. The red lines represent the gates, which open and close during the training and testing phases of the task. For a full description of the delay discounting methodology, refer to section 4.2.3.1.

food cups. The following four days consisted of placing the animal in the stem of the T-maze, and allowing the animal to enter the middle of the arena and choose one arm. Once the animal chose an arm (defined as having placed all four limbs on the base of the arm), the gate behind the animal was closed, and the gate immediately in front of the animal was opened. From there, the animal could then consume the sucrose pellet(s) at the end of the arm within the food cup. During each of these four days the animal was given 5 trials each day, with an approximate inter-trial interval (ITI) of 3 minutes.

The subsequent 12 days of training consisted of two "forced" trials, followed by five "choice" trials. The two forced trials consisted of placing the animal in the stem of the maze, and keeping the entry gate for one arm open, thereby forcing the animal to choose this arm. For each testing day, the first trial had the low reward arm (LRA) opened and the second trial had the high reward arm (HRA) opened. Once the animal entered one of these arms, the arm's first gate was closed and the arm's second gate was opened. Once these forced trials were complete, the animal then had 5 subsequent choice trials wherein the first gate of the HRA and the LRA were both left open and the animal could choose either arm into which to enter.

The 12 days of training were subdivided into three groups (days 1-4, days 5-8, and days 9-12). During training days 1-4, once the animal chose an arm, the arm's first gate would close, and the second gate would open, allowing immediate access to the sucrose pellet(s). During days 5-8 of testing, when the animal chose the HRA, there would be a 5 second delay between the first gate closing and the second gate opening, and during days 9-12 of testing, when the animal chose the HRA, there would be a 10 second delay between the first gate closing and the second gate opening. During this

time there would be no delay between the first gate closing and the second gate opening in the LRA.

The four days of pre-surgical testing were similar to the 12 prior days of training, with the exception of the amount of delay time in the HRA, which was 15 seconds. Again, there was no delay between the first gate closing and the second gate opening in the LRA. The number of HRA and LRA choices made for the choice trials were recorded for each animal for each day.

Post-surgical testing for choice behaviour was performed similarly to pre-surgical testing, and began on PSD7 and continued until PSD24. For the first 6 days of post-surgery testing (PSD7-12), there was a 15 second delay between the first gate closing and the second gate opening in the HRA. For the subsequent 6 days of testing (PSD13-18), there was a 30 second delay between the first gate closing and the second gate opening in the HRA, and for the final 6 days of testing (PSD19-24) there was a 60 second delay between the first gate closing and the second gate opening in the HRA.

4.2.3.2 Ultrasonic Vocalizations

During the testing phases of the delay discounting maze (pre-surgery and post-surgery 15 second delay trials), ultrasonic vocalization recordings (USVs) were obtained when the animal chose the delayed high-reward arm, and analyzed recordings were limited to the 15 second interval the animal would spend between the first and second gates in anticipation of the food reward. The recordings were taken using an Avisoft CM16/CMPA condenser ultrasound microphone (Avisoft, Berlin, Germany) and the

recordings were further processed and analyzed using Avisoft-SASLab Pro software version 5.1 (Avisoft, Berlin, Germany). For the purposes of categorization, USVs were characterized as either 50 kHz “appetitive” calls or 22 kHz “aversive” calls, with calls made with an average mean frequency on or over 30 kHz considered to be an “appetitive” call. This is based on previous work categorizing vocalizations into two separate ranges between 20 - 30 kHz as being "aversive" and 30 - 90 kHz calls as indicating a positive affect (Reno et al. 2013). Animals that did not emit any vocalizations during testing were excluded from further USV analysis, rendering the group sizes for USV analysis to be sham, n = 13; mPFC, n = 10; oPFC, n = 9.

4.2.4 Histology and Infarct Quantification

On PSD 28, rats were anaesthetized with isoflurane and euthanized by decapitation. Brains were removed and immersion fixed in 10% formalin. After 48 hour fixation in formalin, brain tissues were preserved and cryoprotected using Cryomatrix (Thermo Scientific, USA) and stored at -80°C. Brain tissues were sectioned in 50µm segments using a cryostat (Thermo Scientific, USA) and one section every 300 microns was applied to slides, dried for a minimum of 24 hours, and stained with cresyl violet (0.1 % w/v). Images were prepared using a computer scanner (HP Scanjet 7650) at 2400 DPI resolution, and infarct quantification was performed by tracing the area of tissue which displayed a lack of, or abnormal, cresyl violet staining or altered cytological architecture. Tracing was performed using ImageJ software, version 1.45s (ImageJ, National Institutes of Health, USA). Once each area had been traced, the volume of

infarct damage was quantified by summing each individually calculated area and multiplying the area by the distance between each section (300 µm).

4.2.5 Statistical Analyses

Statistical analyses were performed using Graph Pad Prism, Version 5.00 (Graph Pad, La Jolla, USA). Two way repeated measures ANOVA analyses were used to examine the effects of surgical intervention and day of testing on the behaviour of HRA choice, and amalgamated choice data for each day was examined between surgical groups by one way ANOVA. USV data for the number of calls made pre- versus post-surgery were analyzed by converting the number of calls made post-surgery as a percentage of calls made prior to surgery, and each of the values were analyzed by a one-sample t-test with a hypothetical mean of 100%. USV data for the frequency and duration of calls made in the HRA arm was analysed by Wilcoxon signed-rank test comparing each group's pre-surgery vs post-surgery values. Correlation analyses between the size of the ET-1 induced lesion and delay discounting maze performance were analyzed using a Pearson's coefficient. Data was considered significant at $p < 0.05$.

4.3 RESULTS

4.3.1 Delay Discounting

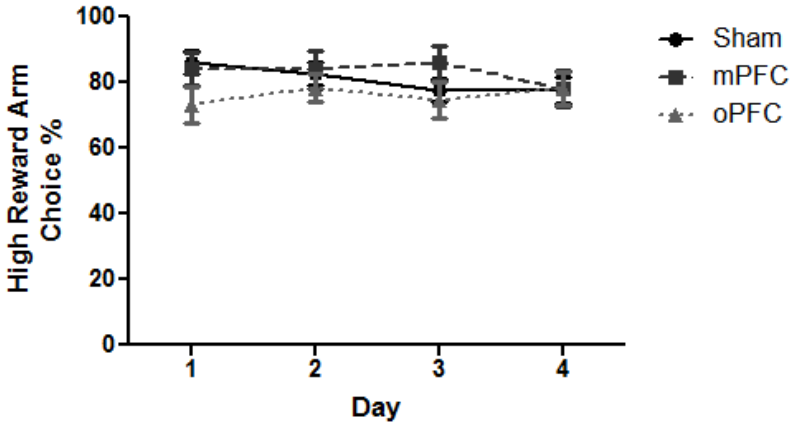
4.3.1.1 Pre-surgery choice behaviour

Task performance within the maze was assessed by determining the percentage of high reward arm (HRA) choices made during the free choice (non-forced choice) portion of the task. As expected, prior to surgery there were no significant differences in task performance between surgical groups ($F_{2,34} = 1.14$ $p > 0.05$) or between days ($F_{3,34} = 0.547$ $p > 0.05$), nor was there any interactive effect between these two variables ($F_{6,34} = 0.888$ $p > 0.05$) (Figure 4.3A). Choice behaviour was also assessed by amalgamating the data from each day for each surgical group (Figure 4.3B) to determine overall choice behaviour, and performing a one-way ANOVA between groups, wherein no difference between groups was found ($F_{2,34} = 1.18$ $p > 0.05$). Average mean HRA choice behaviour for each group was: Sham (80.9% ± 1.93%, n = 16), mPFC (83.0% ± 4.16%, n =10), oPFC (75.7% ± 4.23%, n = 11) (Mean ± SEM).

4.3.1.2 Post-surgery choice behaviour

Task performance was assessed in the same manner post-surgery as it was pre-surgery, wherein HRA choice behaviour percentages were compared between days and between surgery groups (Figure 4.4A). There was no significant difference in choice behaviour over the course of days ($F_{5,34} = 1.19$ $p > 0.05$), nor an interactive effect between surgery group and testing day ($F_{10,34} = 0.584$ $p > 0.05$). However, there was a significant difference between surgery groups ($F_{2,34} = 11.2$ $p < 0.001$). Choice behaviour was also assessed by amalgamating the data from each day for each surgical group (Sham: 71.6% ± 3.00% n = 16; mPFC: 77.3% ± 5.14% n = 10; oPFC: 51.6% ± 3.84%

A.



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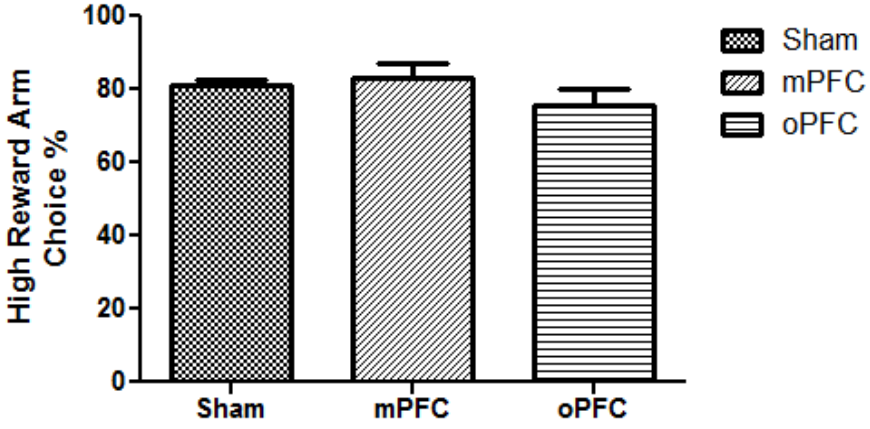
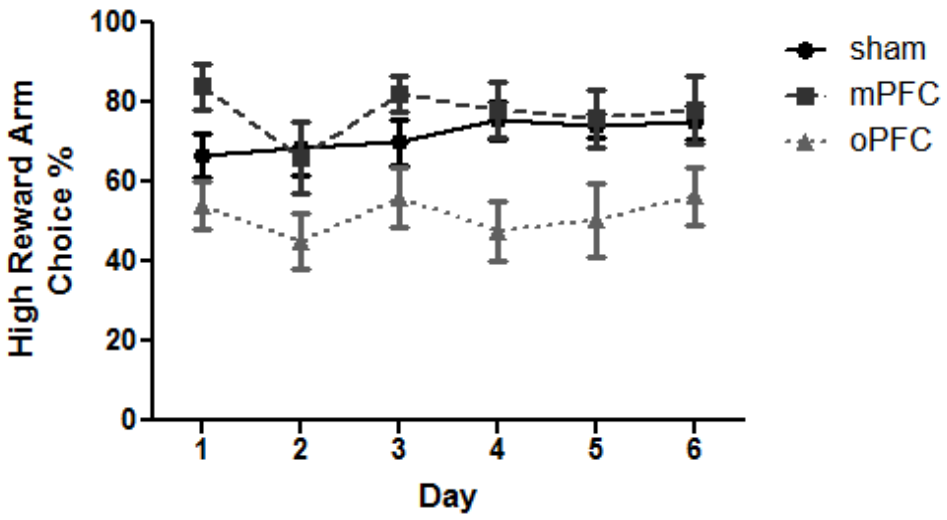


Figure 4.3A and B. Delay discounting choice behaviour pre-surgery. (A) Animals were tested for four consecutive days pre-surgery, in which animals were given two forced choice trials immediately followed by five free-choice trials. There were no significant differences in task performance over days, between surgical groups, nor was there an interactive effect. (B) Amalgamated average pre-surgery choice performance for all three surgical groups. No significant differences were detected between groups. Sham, n = 16; mPFC, n = 10; oPFC, n = 11.

A.



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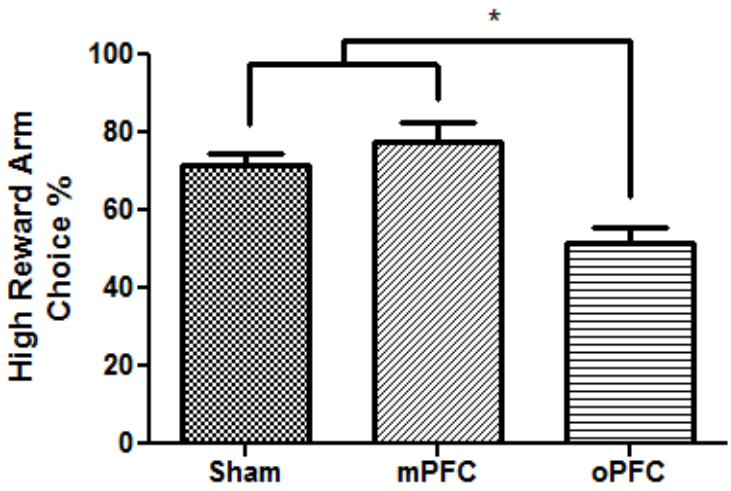
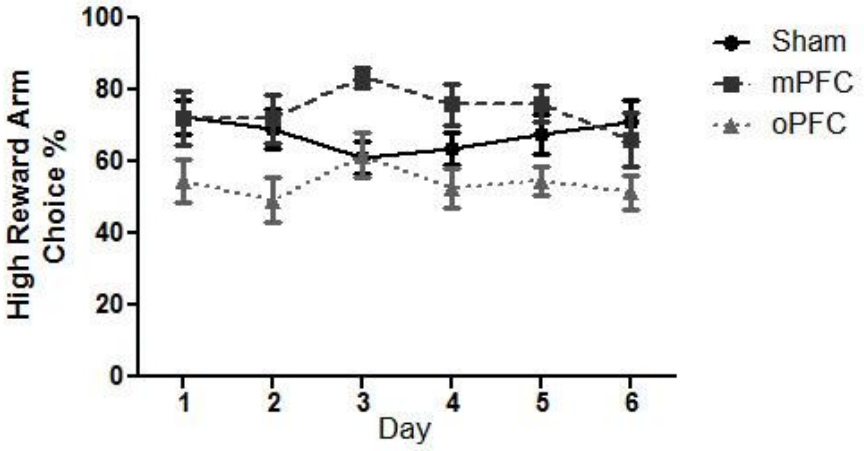


Figure 4.4A and B. Delay discounting choice behaviour post-surgery with a 15 second delay for high reward arm access. (A) Animals were tested for six consecutive days, wherein animals were given two forced choice trials immediately followed by five free-choice trials. There were significant differences in task performance between surgical groups, with no significant effects detected between surgical days nor was there an interactive effect between both variables tested. (B) Amalgamated average post-surgery choice performance for all three surgical groups. Significant differences were detected between the sham and oPFC groups and the mPFC and oPFC groups. Sham, n = 16; mPFC, n = 10; oPFC, n = 11.

n = 11) (Mean ± SEM) and performing a one-way ANOVA and a post-hoc Tukey's Multiple Comparison Test (Figure 4.4B). This revealed significant differences between the sham and oPFC group ($P > 0.01$) and the mPFC and oPFC group ($P < 0.001$), but not between sham and mPFC groups ($P > 0.05$). This analysis was continued for post-surgery behaviour in the delay discounting task for both the 30 second delay and the 60 second delay for the HRA, which revealed some similar trends. For the second delay time of 30 seconds (Figure 4.5A) there was no significant difference in choice behaviour over the course of days ($F_{5,34} = 0.621$ $p > 0.05$), nor an interactive effect between choice behaviour and testing day ($F_{10,34} = 1.30$ $p > 0.05$). However, an effect was found between surgical groups ($F_{2,34} = 7.93$ $p < 0.001$). This effect was further examined, wherein the overall averages from each group over each day were calculated (Sham: 67.6% ± 3.51% n = 16; mPFC: 74.3 ± 3.52% n = 10; oPFC: 54.0% ± 2.74% n = 11)(Mean ± SEM). Statistical analysis revealed significant differences between the sham and oPFC choice behaviour ($P < 0.05$), and between mPFC and oPFC ($P < 0.01$), but none between sham and mPFC ($P > 0.05$) (Figure 4.5B). In the 60 second delay testing (Figure 4.6A), a significant difference was found between surgical groups ($F_{2,34} = 4.63$ $p < 0.05$), and between days ($F_{5,34} = 2.30$ $p < 0.05$), however no interactive effect between these two variables was found ($F_{10,34} = 1.76$ $p > 0.05$). The effect of testing day was further examined by looking at the data for each individual day and determining any differences between the groups through a one-way ANOVA with a Tukey's multiple comparison post-test. There were significant differences between mPFC and oPFC groups on days 1 and 3 ($p < 0.01$), and between sham and oPFC on day 1 ($P < 0 .05$). No

A



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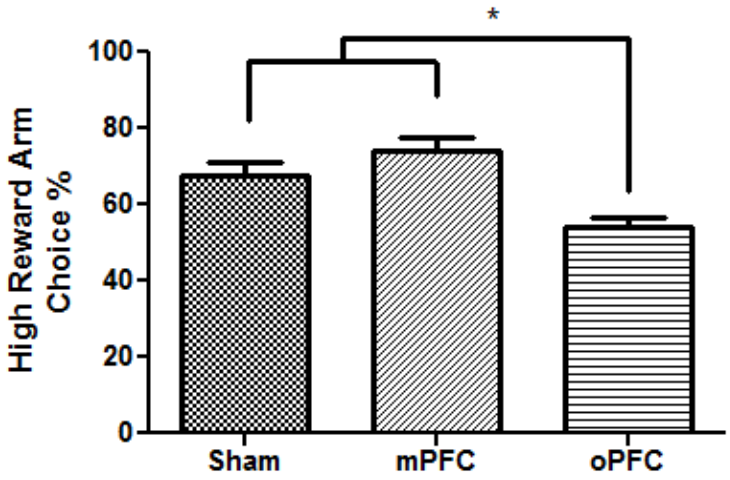
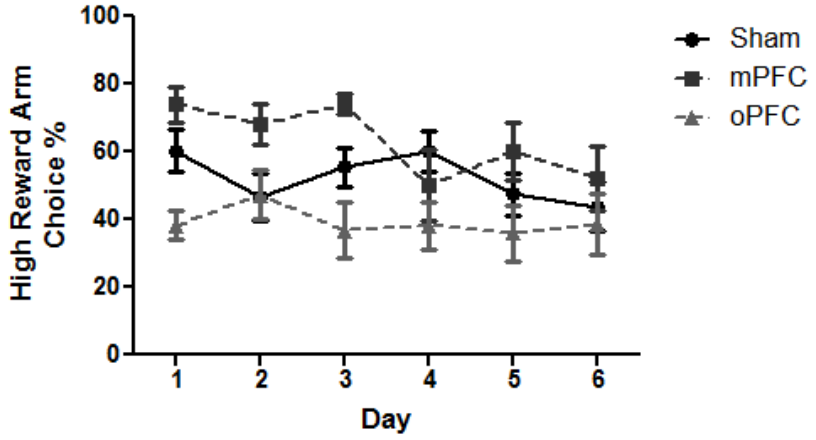


Figure 4.5A and B. Delay discounting choice behaviour post-surgery with a 30 second delay for high reward arm access. (A) Animals were tested for six consecutive days, wherein animals were given two forced choice trials immediately followed by five free-choice trials. There were significant differences in task performance between surgical groups, with no significant effects detected between surgical days or an interactive effect between both variables tested. (B) Amalgamated average pre-surgery choice performance for all three surgical groups. Significant differences were detected between the sham and oPFC groups and the mPFC and oPFC groups. Sham, n = 16; mPFC, n = 10; oPFC, n = 11.

A.



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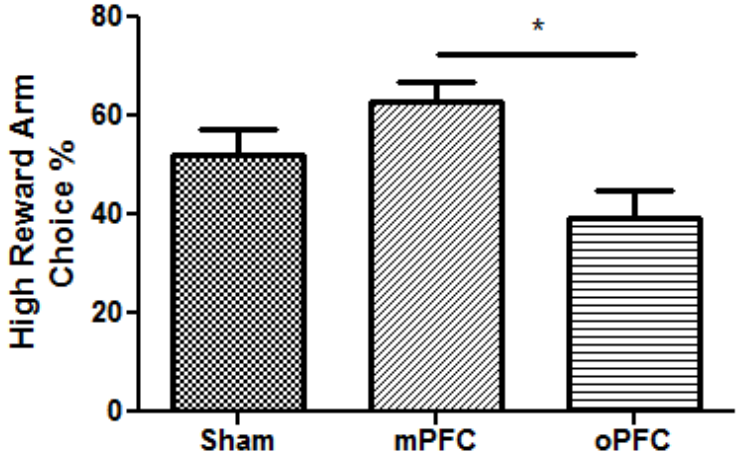


Figure 4.6A and B. Delay discounting choice behaviour post-surgery with a 60 second delay for high reward arm access. (A) Animals were tested for six consecutive days, wherein animals were given two forced choice trials immediately followed by five free-choice trials. There were significant differences in task performance between surgical groups, as well as significant effects detected between surgical days, although there was no significant interactive effect between both variables tested. (B) Amalgamated average pre-surgery choice performance for all three surgical groups. Significant differences were detected only between the mPFC and oPFC groups. Sham, n = 16; mPFC, n = 10; oPFC, n = 11.

differences between mPFC and sham were found. As well, data from each time point (day) for each surgical group were amalgamated and analyzed by a one-way ANOVA with a Tukey's multiple comparison post-test (Figure 4.6B), and a difference was found between mPFC lesioned animals and oPFC lesioned animals ($p < 0.05$), however, there was no difference between oPFC lesioned and sham animals ($p > 0.05$).

4.3.2 Ultrasonic Vocalizations

Three different aspects of ultrasonic vocalizations were examined, analysing the effects of the surgery on pre- versus post-surgery vocalization behaviour. These three variables were the number of vocalizations made, the relative frequency of these vocalizations, and the duration of each vocalization.

The number of 50 kHz calls made during the HRA choice behaviours was measured both pre- and post-surgery and used to calculate the percentage of 50 kHz calls made following surgery. There was no statistical difference between the groups, however, each of the groups was statistically different from a hypothetical mean of 100% ($p < 0.05$). Table 4.3 summarizes the effects of surgery on the number of vocalizations made post-surgery as compared to their pre-surgery values. Although there were no differences between groups as assessed by a one-way ANOVA, each of the groups were individually analyzed through one sample t-tests with a hypothetical mean of 100%, and it was found that each surgical group of animals made significantly fewer calls after surgery as compared to before surgery.

Table 4.3. Percentage of vocalizations made post-surgery, compared to pre-surgery. Although there was no difference in the percent of vocalizations made post-stroke between each of the groups (one-way ANOVA: $F_{2,27} = 0.8218$ $p > 0.05$) there was a significant difference in all groups in terms of the number of vocalizations made as assessed by their pre-surgery values, denoted by a (*) for $p < 0.05$ and by a (**) for $p < 0.01$. Sham, n = 13; mPFC, n = 10; oPFC, n = 9. Values are Mean ± SEM

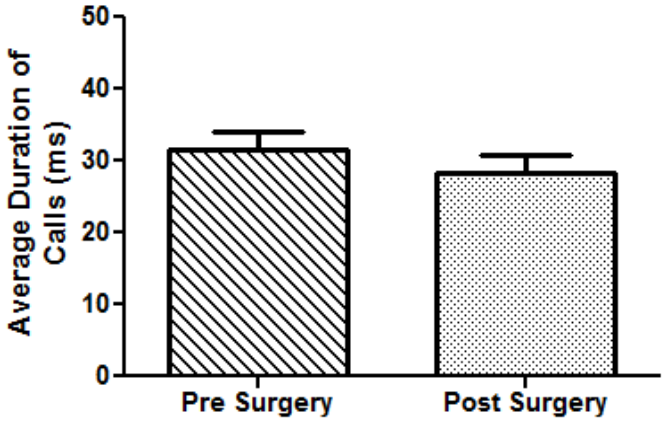
Surgical Group	Percent Pre-Surgery Vocalizations
Sham	**62.2% ± 11.0% ****
mPFC	**47.4% ± 16.8% ***
oPFC	**38.1% ± 14.7% ****

The relative frequencies and durations of individual calls made by the animals were also assessed, examining for differences between their pre- and post-surgery values. Figures 4.7A and 4.7B show the pre- vs post-surgery values for both the duration and frequency of the sham animal calls, which demonstrated no differences post-surgery. Figures 4.8A and B show the pre- and post-surgery values for these same variables in the mPFC lesion group. Unlike the sham group, these animals displayed differences in both duration (Figure 4.8A) and relative frequency (Figure 4.8B) of their calls, making shorter calls of a higher frequency as compared to their pre-surgical values. In contrast, animals in the oPFC lesion group (Figures 4.9A and B), displayed no differences in the relative durations or frequencies of their calls.

4.3.3 Histology and Infarct Quantification

Cresyl violet staining of the anterior portion of the brain relative to bregma showed lesions located primarily within the prefrontal cortex of the animals. Figures 4.10A and B show the extent of prefrontal damage from the two lesion groups. Overall, the damage from the mPFC lesion produced a lesion with a volume of 18.0 ± 2.84(Mean ± SEM). The volume of the assessed damage in the oPFC lesioned animals was 26.1 ± 4.05 (Mean ± SEM). Line graphs in Figures 4.11A and 4.11B give an overall representation of where lesion damage occurred. As assessed by a two-tailed unpaired t-test, there was no significant difference detected in lesion size between the two groups ($t_{19} = 1.60$, $p > 0.05$).

A



B

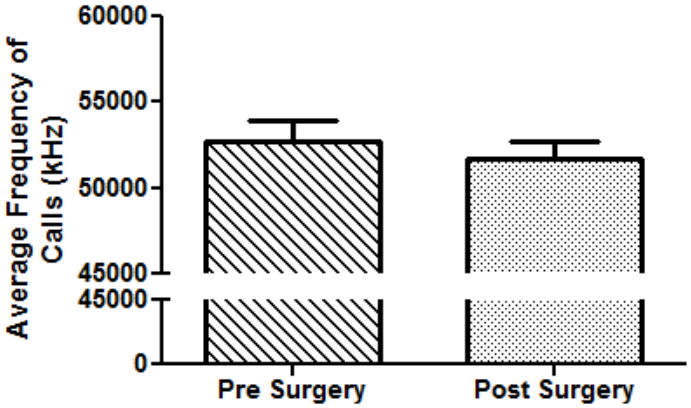
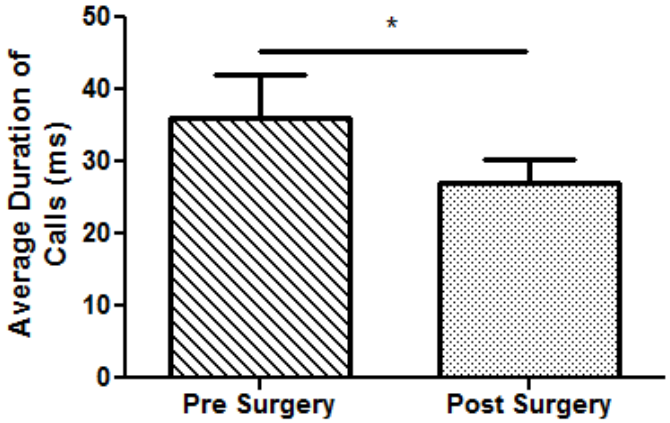


Figure 4.7A and B. Average duration and frequency of ultrasonic vocalizations of sham animals pre- and post-surgery. There was no significant difference detected between pre- and post-surgery values in the (A) average duration of the calls ($p = .340$), or in the (B) average frequency of the calls emitted ($p = .685$). Values are Mean ± SEM, $n = 13$.

A.



B

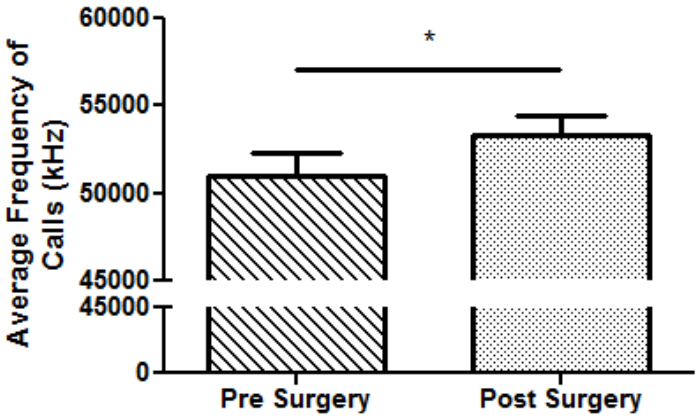
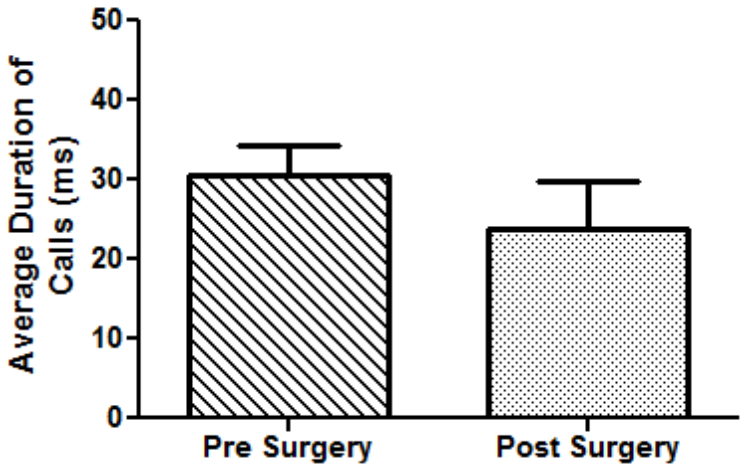


Figure 4.8A and B. Average duration and frequency of ultrasonic vocalizations of mPFC lesioned animals pre- and post-surgery. (A) There was a significant difference in the average duration of the calls made between pre- and post-surgery animals, with post-surgery animals making shorter 50 kHz calls ($p = .048$) (B) There was a significant difference the average duration of the calls made between pre- and post-surgery, with animals emitting higher frequency calls post-surgery ($p = .027$). Values are Mean ± SEM, $n = 10$.

A



B

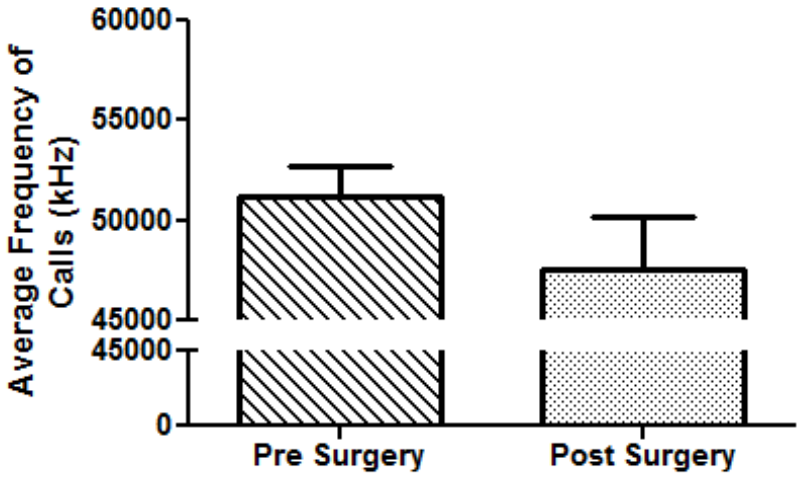
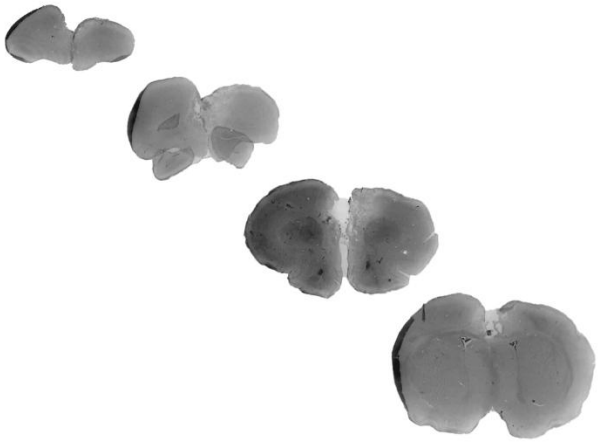


Figure 4.9A and B. Average duration and frequency of ultrasonic vocalizations of oPFC lesioned animals pre- and post-surgery. There was no significant difference detected between pre- and post-surgery values in the (A) average duration of the calls ($p = .359$), nor in the (B) average frequency of the calls emitted ($p = .301$). Values are Mean ± SEM, $n = 9$.

A.



B.

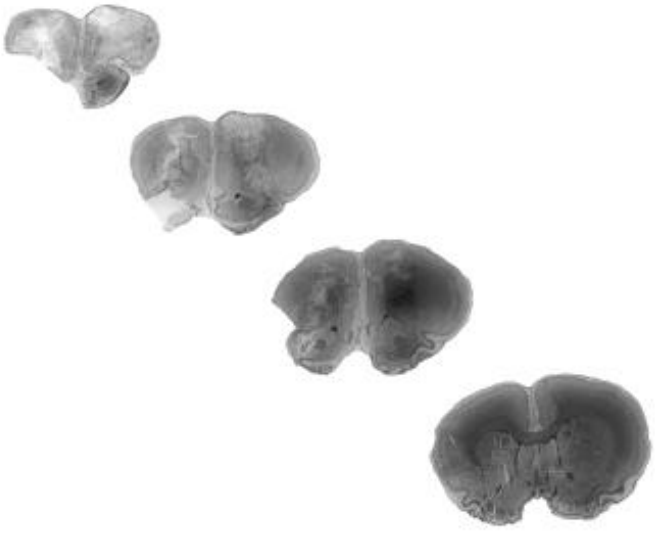
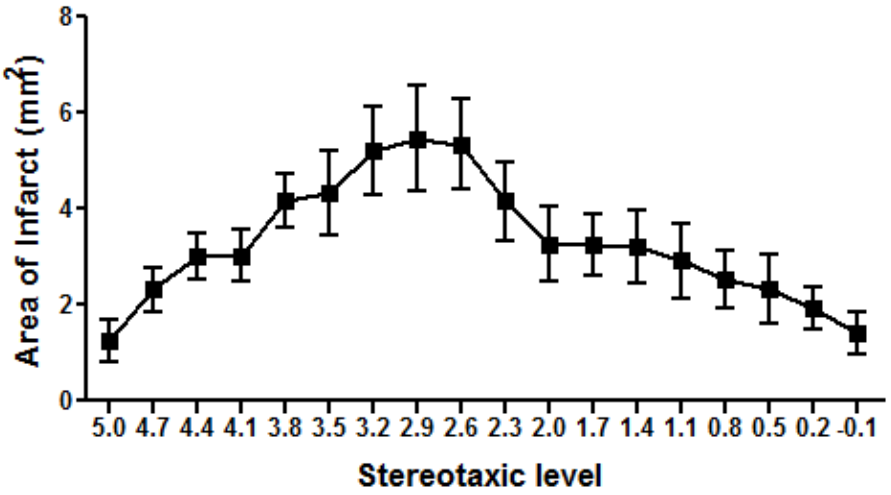
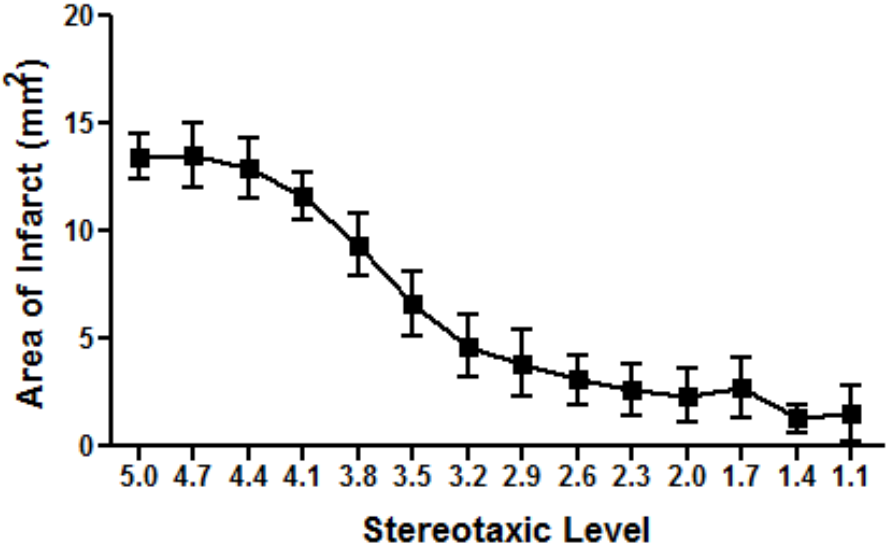


Figure 4.10A and B. Representative cresyl violet staining and sectioning of anterior stroke brain tissue. Damaged tissue is represented by lower stain uptake, as well as altered cytoarchitecture. (A) representative sections of mPFC damaged tissue and (B) representative sections of oPFC damaged tissue.

A.



B.



Figures 4.11A and B. Line graphs representing the approximate areas of damage and size of areas damaged as a result of the ischemic lesions.(A) area damaged as a result of the mPFC lesion (n = 10) and (B) area damaged as a result of the oPFC lesion (n = 11). Values are Mean ± SEM.

Further analysis comparing the relative size of the infarct within the mPFC group and delay discounting choice behaviour revealed no correlation between lesion size and HRA choice behaviour at any time point tested (15 second choice behaviour [$r = .2830$, $n = 10$, $p > 0.05$]; 30 second choice behaviour [$r = -0.064$, $n = 10$, $p > 0.05$]; 60 second choice behaviour [$r = -0.047$, $n = 10$, $p > 0.05$]). The same was found when comparing oPFC choice behaviour and lesion size, wherein no correlation was present between lesion size and HRA choice behaviour (15 second choice behaviour [$r = -0.017$, $n = 11$, $p > 0.05$]; 30 second choice behaviour [$r = -0.125$, $n = 11$, $p > 0.05$]; 60 second choice behaviour [$r = -0.097$, $n = 11$, $p > 0.05$]).

4.4 DISCUSSION

Part of the dilemma for research into post-stroke cognitive dysfunction is the lack of cognitive deficit models for pre-clinical trials, especially with regards to executive functions. Currently, there are experimental models of stroke focusing on its effects on depression and other cognitive ailments post-stroke, but there are few models that display executive dysfunctions including attentional deficits, inhibitory control, and proper decision making.

The present study tested the effects of focal ischemic lesions localized to the mPFC and oPFC on delay discounting behaviour and ultrasonic vocalization behaviours. It was found that oPFC lesions had very specific effects on delay discounting choice behaviour, and that mPFC lesions appeared to have no effect relative to control animals. This finding is similar to what was found by Rudebeck et al. (2006), wherein animals

with quinolinic acid lesions to the oPFC were significantly worse at the delay discounting task than control animals and animals with lesions to the anterior cingulate cortex (ACC). Other studies examining this effect found that oPFC lesioned animals displayed similar impulsive behaviours in an operant conditioning paradigm (Mobini et al. 2002). In contrast to these findings, other research has found that the role of the oPFC in delay discounting may be slightly more nuanced. A further paper examining oPFC lesions in delay discounting found no differences in post-lesion inhibitory behaviour in a non-spatial version of the task (Mariano et al. 2009), and other previous work by Winstanley et al. (2004) suggested the oPFC is not required for inhibitory control, and that basal lateral amygdala lesions contribute to increased impulsive choice behaviour rather than oPFC lesions. These differences could be explained by differences in the behavioural testing paradigm employed, because oPFC lesions may be affecting specific aspects of inhibitory control. Another explanation could be that the lesions produced near the oPFC could have affected circuits between this region and the hippocampus, which have also been directly implicated in proper inhibitory control (Chudasama et al. 2012).

In the current study, lesions to the mPFC appeared to have no effect on inhibitory control behaviour. Like oPFC lesions, the effects of mPFC on impulsivity appear nuanced. There have been some studies that have found a link between the mPFC and inhibitory control under certain circumstances (Winstanley 2005; Loos et al. 2010), and others finding no such link (Eagle and Robbins 2003; Eagle et al. 2007). These differences may depend on the circumstances of the experimental manipulation of the test animal, as well as the behavioural testing procedure employed. As well, a paper

by Feja et al (2014) demonstrated that inhibitory behaviour as measured by a five-choice serial reaction task was altered by inactivation of the vmPFC, but inhibitory control as measured through delay discounting was not affected. Therefore, our model may not have demonstrated measureable deficits in inhibitory control in this delay discounting maze, however, future experiments using this model of ischemic stroke could test inhibitory control in different behavioural tasks.

One issue with the behavioural testing that was not addressed by our delay discounting paradigm was handedness. Although rats do not display as much of a right-handed bias as humans, it has been noted that rodents' circling behaviour and forepaw use, measured through an operant conditioning chamber, are right side biased (Glick and Ross 1981). Future studies with this paradigm would be able to control for this by testing the animal's innate preference for sides both before and after surgery, as brain lesions have been shown to affect handedness in response to lesions, depending on pre-lesion behavioural training (Peterson and Devine 1963).

The overall size of the oPFC lesions was comparable to the mPFC lesions in terms of overall volume (17.98 mm^3 in mPFC animals versus 26.05 mm^3 in the oPFC animals), although there was a tendency for the orbital lesions to be slightly larger in size. This may be due, in part, to the bilateral location of the imposed lesions. For the mPFC animals, the sites of injection for the vasoconstrictor ET-1 are approximately 1.4 mm apart (M/L -0.7 and M/L +0.7 from the midline). The drug's effects on the surrounding tissue appear to extend from the site of injection and overlap with the other bilateral injection. For the oPFC lesioned animals, there is very little to no overlap in the bilateral lesions, with the lesions spaced 2mm away from the midline; 4 mm apart.

Another interesting finding in the oPFC group was that the greatest extent of lesion size was not found at the site of injection (4.0 mm anterior to bregma), but rather closer to the anterior portion of the brain. This was not the case for the mPFC group in our study and in our previous study (Deziel et al. 2015; see Chapter 3), wherein the maximal size of the lesion was at the site of injection, 3.0 mm anterior to bregma. This could be explained by incorrect injection coordinates, however sham animals with aCSF injected into the oPFC showed needle tracts entering into approximately the appropriate areas of the oPFC (Figures 4.12A - C). An alternate explanation could be that the degree of bevelling of the Hamilton Removable Needle, combined with the direction of the needle hole facing the anterior pole of the brain. During surgery, the location of the needle hole faces the anterior end of the brain, which could have the effect of pushing the injection liquid forward. Provided that there is less interstitial tissue to displace, compared to the medial PFC, the displacement of the injection liquid could potentially move further forward compared to the medial PFC injections. Future experiments could determine whether the bevelling of the needles had an effect by either using flat needles, or by having the hole of the needle facing the posterior of the brain and observing the ET-1 lesion's size and location.

Other future studies examining these two different stroke lesion models may wish to examine the lesions' effects on post-stroke depression. The prefrontal cortex is an area of the brain that has been directly implicated with depression and depressive symptoms (Koenigs and Grafman 2009) , and there have been numerous links made between the co-morbidities of depression and executive dysfunction (Snyder 2013; Letkiewicz et al. 2014). Although there are models of post-stroke depression with some

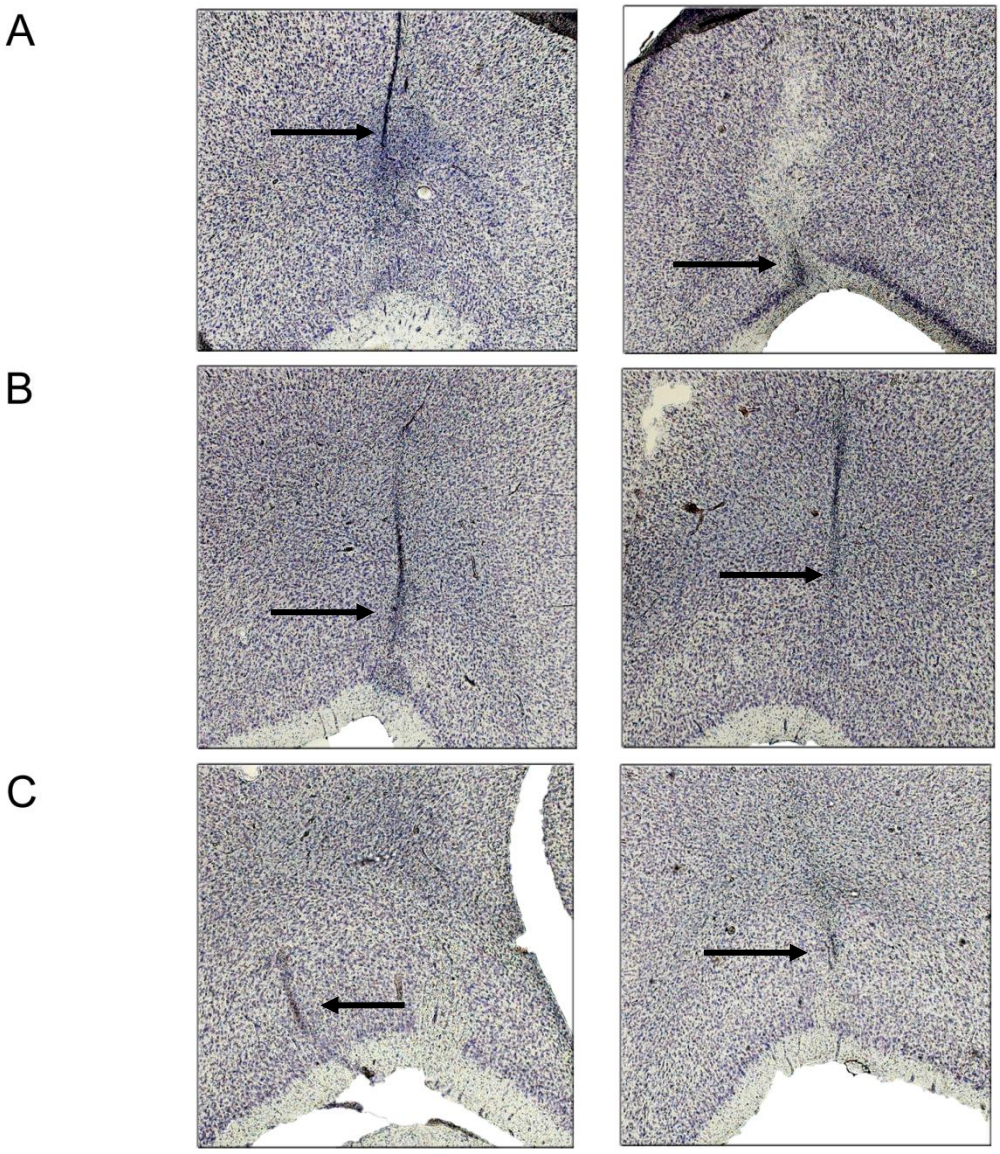


Figure 4.12 A-C Needle injection points in three selected sham animals. Injections in these animals occurred between approximately +4.2 - +4.5 A/P of bregma.

measureable cognitive dysfunction in certain domains, none of these models test the effects of the stroke on executive function (Kim et al. 2015; Wu et al. 2015). Understanding the underpinnings of concurrent executive dysfunction and post-stroke depression could be aided by using focal ischemic models of stroke which display both symptoms.

The discrepancies between behaviour and changes in USV production were counterintuitive, as it would be expected that changes in the animals' behaviour would be reflected by changes in their vocalizations (i.e. decreased choice of the high reward arm may result in changes to 50 kHz calls, as those indicate a positive affective state). However, it was the mPFC lesions, not the oPFC lesions, that resulted in changes to vocalization frequencies and durations.

These differences may be explained by examining which cognitive processes are involved with the control of affective states, rather than those responsible for inhibitory control as measured through a delay discounting paradigm. For example, it has been shown that mPFC lesions affect both physiological stress responses as well as behavioural stress responses, both of which indicate that lesions specific to this area of the brain may be anxiolytic (Sullivan and Gratton 1999; Shah and Treit 2003). If the animals in the mPFC group have a differential response to stress compared to their sham and oPFC counterparts, the differences in vocalizations could be a result of these changes. Further research should be performed to further sub-categorize these 50 kHz calls, which has been done previously in other paradigms, including studies of the effects of social interaction, pharmacological therapies, and food cue changes in rat vocalizations (Wright et al. 2010; Buck et al. 2014).

Although the differences in USV production in these animals are interesting, only adult male Sprague-Dawley animals were tested in these experiments. Previous experiments have noted some strain differences in rat ultrasonic behaviour, with differences in vocalizations in certain rat strains observed as early as post-natal day 6 (Sales 1979). However, research into different USV behaviours in adult rat strains is sparse, and it is unknown whether different strains would respond in similar or dissimilar ways. As well, there have been noted differences between male and female rat vocalization exchanges, however these experiments are often performed to test for vocalizations made between male and female animals during courting or copulation (McGinnis and Vakulenko 2003), and it is unknown whether female animals would vocalize differently in our experiments in response to food rewards as appetitive stimuli.

In conclusion, the experiments described in this chapter demonstrate that ischemic lesions localized to the prefrontal cortex have a multitude of effects on certain cognitive functions. Future studies regarding these models of post-stroke cognitive changes could open new avenues to research into pre-clinical stroke assessment, and should attempt to better characterize the changes to inhibitory control and appetitive vocalization patterns of these animals.

CHAPTER 5

GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1 SUMMARY

As discussed previously in this thesis, stroke is one of the most common causes of death and disability worldwide (Kim and Johnston 2011; Lozano et al. 2012). People can live a relatively long time post-stroke, and a significant portion of these individuals are left with debilitating injuries that affect motor, sensory, and cognitive function. Related to these disabilities, we currently have fairly robust models of motor dysfunction post-stroke that accurately portray the behavioural and pathophysiological effects of the disease, as well as animal models of ischemia displaying some limited aspects of cognitive dysfunction. Unfortunately, there is a distinct lack of post-stroke models to study higher-order levels of cognitive dysfunction that makes it difficult to identify and test the effects of different pharmacological and behavioural therapies as potential treatment strategies to conserve or rehabilitate cognitive functionality lost due to an ischemic stroke. Because of the large worldwide prevalence of this disease, the increasing number of surviving stroke patients in the developed and developing world displaying cognitive dysfunction, and the relative lack of animal models of higher-order cognitive dysfunction, the experiments described in this thesis were performed in an attempt to model cognitive dysfunction in this disease in rodents.

The work contained within this thesis gives an overview of the histological effects of focal ischemic lesions within the prefrontal cortex, an area intricately linked to higher-order cognitive function, as well as the results of behavioural tests designed to test certain higher-order cognitive functions and other behaviours in the rat. Initial development of the model began as discussed in chapter 2, wherein two separate

experiments were performed, the first of which was to test particular injection protocols in order to target damage to the mPFC of the rat. The second set of experiments was performed to discern what sorts of behavioural effects these lesions might have on the animal. The first experiments examined a four-injection versus a two-injection endothelin-1 protocol, and determined that a two injection protocol within the mPFC results in a more focused lesion as well as less animal mortality. The protocol was then tested in a larger experiment with sham animals undergoing the surgery with a vehicle (aCSF) injection, and test animals injected with one set of bilateral ET-1 injections to the mPFC. These animals were tested in an elevated plus maze as well as a task examining temporal object memory. In these experiments, it was found that lesions affecting the mPFC produced anxiogenic behaviours in the animals as assessed through time spent in certain arms of the EPM as well as the performance of particular behaviours while in the maze itself. The temporal object recognition task was ineffective at assessing temporal order memory changes in the animals, and it was hypothesized that this observation could have been due to methodological discrepancies between the test used in this experiment and others that have been performed in the literature (Hannesson et al. 2004; Hotte et al. 2005).

The next series of experiments, discussed in chapter 3, with the ET-1 two injection model were further developed to test for specific deficits in executive function in the domain of attention, with a specific focus on set-shifting. As well, experiments were performed to modify the temporal object recognition task in order to elucidate potential changes to temporal order memory. In the experiments performed in this chapter, it was noted that animals with damage to the mPFC had some specific

difficulties in switching attention from colour features in the set-shifting maze to texture features, as well as making considerably more perseverative errors while in the set-shifting task. Unfortunately, once again the temporal object recognition (TOR) task failed to be effective at measuring temporal order memory in these animals despite the methodological changes made from the previous experiments.

Further cognitive testing of the two injection model continued for the next series of experiments (chapter 4), examining the effects of two separate lesion sites (the oPFC and the mPFC) on decision making and inhibitory control as assessed through a delay discounting task, as well as examining the effects of lesions on the production of ultrasonic vocalizations while running the maze. It was found that oPFC lesions, rather than mPFC lesions affected the behaviour by having the animals choose the smaller, but immediately available, reward over the higher delayed reward. Interestingly, mPFC lesions, but not oPFC lesions, affected certain aspects of appetitive 50 kHz vocalizations, indicating that the changes in behaviour did not necessarily correlate to changes in appetitive vocalizations and vice versa.

Many of these experiments provided results that were not originally hypothesized to occur, leading to further questions regarding the models themselves as well as ideas for future experiments. These will now be discussed.

5.2 THE ENDOTHELIN-1 ISCHEMIC MODEL AND EXECUTIVE DYSFUNCTION: CONSIDERATIONS

In general, there are two large categories by which we can characterize rodent models of ischemia: global models and focal models. Global models function by occluding the vertebral or carotid arteries, mimicking a cardiac arrest. Focal models, on the other hand, function by occluding smaller vessels directly in the brain, and are thought to be more representative of strokes found in human patients as they replicate the conditions of the disease more closely than global ischemic models (Tajiri et al. 2013).

The post-stroke cognitive dysfunction models developed during the course of this thesis used the compound endothelin-1 to mimic the effects of focal ischemic stroke in human patients. ET-1 has been used for three decades in many different animal models of stroke, although the first use of ET-1 that had the direct intention of specifically affecting cognitive function was through the application of the compound to the anterior cerebral artery of the rat, performed by Ward et al. (1998). In this paper, it was noted that these ischemic lesions affected the ability of rats to accurately perform the serial reaction task as well as choice reaction tests, indicating deficits in the initiation of movement which left motor function intact. Recently, other groups have attempted to replicate the effects of ET-1 ACAo lesions, having some success in affecting aspects of executive function including decision-making, spatial working memory, and some exploratory activity (Endepols et al. 2015). Other uses of ET-1 for inducing cognitive dysfunction have been described in rats, wherein ET-1 was injected directly next to the

MCA, although the types of cognitive dysfunctions tested for concentrated mainly on spatial memory and working memory (Jiwa et al. 2010). Because of these experiments and the previous use of ET-1 in our lab to induce focal ischemic lesions directly in to the motor strip (Livingston-Thomas et al. 2014), it was hypothesized that injecting this compound directly into areas of the prefrontal cortex could be an effective method of mimicking ischemic stroke affecting higher-order cognitive functions.

Through the experiments done during the course of this thesis, it was demonstrated that, overall, there were a number of positive benefits to using this ischemic stroke model. First, the use of ET-1 to induce the lesions demonstrated that it could be targeted to specific brain areas with a minor amount of damage to other areas outside of the region of interest. Secondly, the surgical procedure was relatively easy to accomplish and resulted in few animal mortalities. Although, from an animal welfare standpoint this is important, it is most crucial to the work in this thesis because of the small number of animals in each cohort for each study: reducing the number of animals in the experimental group could increase the variability found in the behavioural tests. Finally, and of most relevance to this thesis, is that the lesions *do* result in some measurable forms of post stroke cognitive dysfunction as previously described in Chapters two through four. These forms of cognitive dysfunction include deficits in set-shifting, changes in exploratory behaviour, increases in anxiogenic behaviours, and altered decision-making. Despite some of the negative aspects of the model, including a small amount of damage outside of the targeted lesions of the medial or orbital prefrontal cortex as well as the appearance of aggressive behaviours directed towards

the experimenters, the model appears to be suitable for the study of some executive function deficits post-stroke.

Although the model did display some of the hypothesized deficits in cognitive function, there are some limitations to using this method in an attempt to model stroke. For one, Localized lesions in human patients, specifically within the PFC, are relatively rare. Although many patients have deficits in executive function, it is more than likely not due to a lesion localized to the PFC. As well, in many patients that have suffered a stroke they may have coinciding deficits in both cognitive and motor functions. In the ET-1 stroke models assessed in this thesis, the animals were not tested for motor dysfunctions. Therefore, it is unknown whether motor dysfunction could have had an effect on some of the cognitive tests employed.

If the ET-1 model was to be continued for further use of modelling executive dysfunction post-stroke, there are other areas of the cortex that could be targeted to elicit changes in higher order cognition. For example, it has been noted that damage to white matter, as measured through MRI, is correlated to reduced executive function as well as episodic memory (Smith et al. 2011), which could provide another avenue for the study of executive dysfunction in rats. It has also been noted that damage to thalamic structures also leads to some measurable deficits in executive function (Carrera and Bogousslavsky 2006). Finally, it should be noted again that a large percentage of strokes that occur in human patients have occlusions of the middle cerebral artery. Therefore, it may be reasonable to revisit the ET-1 MCAo model and to examine it thoroughly for any signs of executive dysfunction which could be measured through tests of attention, decision making, or inhibitory control.

5.3 ULTRASONIC VOCALIZATIONS AND THE PREFRONTAL CORTEX: CONSIDERATIONS

An interesting side aspect of the fourth chapter of this thesis examined the effects of mPFC and oPFC lesions on ultrasonic vocalizations (USVs), and found some unexpected results. Originally, what was predicted for this experiment was post-surgery that animals with ET-1 induced lesions would produce fewer 50 kHz USVs as well as produce more 22 kHz USVs. This was hypothesized as damage to the PFC can affect emotionality and may cause animals to become more aggressive or anxious, thereby reducing the number of appetitive 50 kHz calls and thus increasing aversive 22 kHz calls.

Although there was some evidence supporting the first prediction in that mPFC and oPFC surgical procedures did reduce the number of 50 kHz calls, which has been seen before with lesions to the mPFC (Frysztak and Neafsey 1991), it appeared that sham animals also reduced their 50 kHz vocalizations. This would indicate that the reduction of calls was due to the surgical procedure itself, rather than the extent and nature of the ET-1 lesions themselves. As well, animals overall made very few 22 kHz calls overall, with most never making one individual aversive call throughout the entire series of experiments, and there was no evidence of any of the groups increasing the number of aversive calls post-surgery. Unexpectedly, the mPFC lesioned animals appeared to be modulating their calls, which was demonstrated by the animals changing the relative frequency and duration of their calls to emit higher pitched and shorter

vocalizations. Although there is evidence that rodents have sub-categorizations of 50 kHz calls (Brudzynski 2013), it is unclear from the data obtained during the experiments conducted in this thesis if the animals are changing to specific subtypes of calls.

The lack of 22 kHz calls emitted by the animals was surprising. However it has been noted that 22 kHz calls are often elicited by overtly negative stimuli, such as predators or chronic pain (Blanchard et al. 1991; Calvino et al. 1996), rather than a lack of positive stimuli. If future experiments were planned to examine the effects of ischemic lesions specifically on some aspect regarding 22 kHz calls, negative stimuli designed to elicit these calls should be used.

5.4 FUTURE DIRECTIONS

Arising from the results of the experiments presented within this thesis, there are a number of methodological modifications and future experiments that could be made to better refine and further the discoveries made in this work.

5.4.1 METHODOLOGICAL CONSIDERATIONS

One of the first changes that could be made regarding this work could be to implement a plan for housing the animals in groups rather than housing the animals permanently in separate cages, as was done throughout the thesis. There has been evidence that periods of isolation housing can have negative effects on certain behaviours, including affecting reversal learning in adolescent rats (Han et al. 2011),

causing memory deficits and changes in object recognition behaviour (Bianchi et al. 2006), and affecting auditory gating as measured through pre-pulse inhibition (Stevens et al. 1997). As well, it has been noted that pre-stroke social isolation in human patients affects functional recovery post-stroke, indicating a potential link between social interaction and the negative effects of ischemia (Boden-Albala et al. 2005).

Despite the potential positive effects of group housing, by the nature of the surgery itself and the types of lesions induced in the animals it may not be completely feasible for forced social interaction immediately following the surgical procedure. Animals recovering from stroke would need an undetermined period of recovery time prior to reintegration, and the effects of mPFC or oPFC ischemic lesions could alter the social interactions between the animals, potentially rendering the animals more aggressive (Kolb and Nonneman 1974), causing harm to the animals housed together. If the animals were to be housed in groups, a pilot study would need to address these issues first before continuing with a more in-depth study.

Prior to experiments, power analyses to determine the number of animals needed for each of the experiments were not conducted. Although there were statistical differences in behaviour found within some of the experiments, using a larger subset of animals may have revealed further differences that could have been justified depending on the estimated effect of the lesion of behaviour. Relating to this, the effect size of the lesions upon cognitive behaviour, although statistically significant, was relatively small. Future experiments examining intervention strategies to alleviate these cognitive deficits may need to use larger groups of animals to be able to discern whether treatments have any significant effects.

The lesions presented in this thesis are induced into both sides of the cortex (bilateral), as opposed to ischemic strokes in human patients which typically present unilaterally (Grotta et al. 2015). The reasoning behind performing the surgeries bilaterally was to increase the likelihood of observing post-stroke deficits in function by injuring both regions of interest in each hemisphere. However, recent evidence has demonstrated that unilateral lesions in the prefrontal cortices of rodents can induce measureable deficits in some cognitive domains (de Souza Silva et al. 2016; Vahid-Ansari et al. 2016). Therefore, if further experiments examining the effects of prefrontal lesions on higher order cognitive function are continued, it is suggested that they are to be initially performed unilaterally. If no effects are seen with a unilateral infarct, then a bilateral infarct should be performed. Another methodological consideration would be the use of older animals versus younger adult animals as was done in this thesis. It has been noted that older animals may be more representative of the disease found in human patients (Casals et al. 2011), and it has been recommended that pre-clinical stroke trials take advantage of older animals (Fisher et al. 2009). It is unknown what the effects of ET-1 would be in aged rats, however, therefore further pilot studies would need to determine the overall lesion size and survivorship of older rats undergoing this procedure.

The use of butorphanol, although commonly used as a post-operative analgesic in rodent models (Zhu et al. 2004; Ikeda et al. 2015), may have caused unintended consequences within our model. Butorphanol has been known to affect behaviour, including increased food intake as well as increased locomotor activity (Levine and Morley 1983; Liles and Flecknell 1992), and opioids are known to affect body

temperature (Adler et al. 1988), which has been shown to affect both lesion size and functional deficits post-stroke (Green et al. 1992; Clark et al. 2008). However, of the many commonly-used post-operative opioids, butorphanol appears to have the smallest analgesic effect and the shortest analgesic time, thereby limiting this potential confound as best as possible (Gades et al. 2000).

Finally, another aspect that should be included with future research with this model would be to include some basic tests of motor function post-stroke. Although most of the executive function tasks employed within this thesis would not have been affected by slight motor dysfunction, tests of object recognition requiring object exploration could be affected by altering the amount of time spent exploring objects as a whole. If future cognitive tasks requiring proper motor function were employed, assessing motor capabilities would be recommended.

5.4.2 Proposed Future Experiments

One of the first sets of experiments performed in Chapter 3 concerned the effects of mPFC lesions on set-shifting, finding that under certain parameters this behaviour appears to show deficits in lesioned animals versus sham animals. What was interesting is that it was noted that animals shifting to particular cues of the maze, specifically from colour to texture, appeared to have more difficulty learning to associate this feature with the receipt of a food reward. In contrast, lesioned animals shifting from texture to colour cues performed as well as control animals. What is different from other experiments that have investigated the effects of mPFC manipulations on set-shifting behaviour is that

this difference in colour / texture set-shifting has not been observed (Birrell and Brown 2000; Stefani et al. 2003). At this time, it is unclear whether this difference is due to material and methodological differences between our study and others performed, or because the nature of these ischemic lesions in the mPFC is intrinsically different than those induced through other methods. Although this method of testing set-shifting is well established (Stefani et al. 2003), there are other methods of testing set-shifting in rodents. For example, a modification of this maze demonstrated by Floresco et al. (2006) using a strategy set-shifting paradigm appeared to be sensitive to manipulations within the mPFC of rats. Employing this methodology could be one further method to reassess the attentional set-shifting abilities of these animals to further understand the true nature of the deficits induced by these lesions.

One aspect of post-stroke cognitive dysfunction that has been extensively examined in the literature has been post-stroke depression, which can affect significant portions of the population (Paolucci 2008). Interestingly, post-stroke depression has been correlated to increased levels of executive dysfunction, indicating that there may be some connection between these two phenomena (Snyder 2013). At present, most methods to induce this in rodents rely on both the induction of ischemia as well as another factor, such as subjecting the animal to chronic mild stress (Wang et al. 2009), however, with targeted lesions to regions associated with post-stroke depression it may be possible to cause PSD without the need for further experimental manipulations. For example, depression has been associated with experimental manipulations affecting the nucleus accumbens and the olfactory bulbs (Di Chiara et al. 1999; Song and Leonard

2005), therefore targeted lesions to these or other regions may be able to elicit the PSD phenotype without further intervention.

Finally, follow up studies could examine the effects of mPFC lesions on the specific types of USVs produced pre- and post-lesion. Although adult USVs are generally classified as either 22 kHz (aversive) or 50 kHz (appetitive) calls (Schwarting and Wöhr 2012), the latter of these two calls can be further sub-categorized in a multitude of different calls (Wright et al. 2010). Further knowledge of the types of appetitive calls emitted by lesioned animals may give future insights as to the exact nature of the large number of 50 kHz calls produced by rodents.

5.5 CONCLUSION

Despite decades of research into pharmacological and behavioural therapies for ischemia as well as animal models of the disease, we are still unable to provide much in the way of targeted therapies for post-stroke cognitive dysfunction. Of course, this is in part due to the massive complexity and fragility of the human central nervous system, but some of this failure is due to a lack of effective modelling of the post-stroke cognitive dysfunction that is common amongst surviving stroke patients. I believe the information derived from the experiments presented in this thesis provides valuable information for other researchers attempting to model post-ischemic cognitive dysfunction as well future directions for experiments designed to aid in the treatment of this disease.

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APPENDIX A: aCSF COMPOSITION

Compound	g/mol	Conc (mM)
NaCl	58.44	124
KCl	74.55	4
NaH2PO4	137.99	1.24
MgSO4	246.48	1.3
CaCl2	147.01	2
NaHCO3	84.01	26
Glucose	180.2	10

pH of aCSF was adjusted to 7.4 using hydrochloric acid.